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DIFFERENTIAL REGULATION OF CA^{2+} SIGNALS IN DOPAMINE NEURONS: A POTENTIAL MECHANISM FOR NEUROADAPTIVE CHANGES UNDERLYING DRUG ADDICTION

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by

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Dedication

This dissertation is dedicated to my wife Weiting Zhang, my mom Shude Jin, my sister Huiyu Li, and my niece Xiuyan Jin.

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DIFFERENTIAL REGULATION OF CA^{2+} SIGNALS IN DOPAMINE NEURONS: A POTENTIAL MECHANISM FOR NEUROADAPTIVE CHANGES UNDERLYING DRUG ADDICTION

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A key adaptive change in the brain reward circuitry during the development of drug addiction is augmented dopamine (DA) release in response to addictive drugs. Potentiated glutamatergic synaptic transmission onto midbrain DA neurons has been suggested to be one of the cellular mechanisms mediating this change. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) rise associated with postsynaptic bursts of action potentials (APs) and metabotropic glutamate receptor (mGluR) activation has been implicated in the induction of long-term potentiation (LTP) and long-term depression (LTD), respectively, of glutamate transmission in DA neurons. In this dissertation, we found a unique mechanism

that differentially regulates these two opposing Ca^{2+} signals. We performed patch-clamp recordings from DA neurons in acutely cut brain slices, and showed that tonic activation of metabotropic neurotransmitter receptors (such as mGluRs, α1 adrenergic receptors, and muscarinic acetylcholine receptors), attained by weak, sustained (~1 sec) synaptic stimulation or bath application of selective agonists, augmented AP-induced Ca^{2+} transients while inhibiting Ca^{2+} signals elicited by strong, transient activation of mGluRs. This differential regulation is mediated by increased intracellular inositol 1,4,5-triphosphate (IP_3) levels, since it was blocked by IP_3 receptor antagonist heparin and reproduced by photolytic application of IP_3. We further showed that AP-induced Ca^{2+} transients were regulated by the firing context of dopamine neurons. Evoking APs repetitively at low frequency (2 Hz) mimicking the basal firing of DA neurons caused inactivation of IP_3 receptors and inhibited AP-induced Ca^{2+} transients. IP_3 facilitation of single AP-induced Ca^{2+} signals was completely abolished during the AP train, while facilitation of Ca^{2+} signals triggered by bursts of APs (5 spikes at 20 Hz) was attenuated by less than half, indicating that increased IP_3 level selectively amplifies Ca^{2+} signals associated with bursts but not single APs in a tonicly firing neuron. Finally, we obtained evidence suggesting that psychostimulant amphetamine may augment burst-induced Ca^{2+} signals via both depression of basal firing and production of IP_3. We propose that the differential Ca^{2+} regulation mechanisms described in this dissertation may induce a shift in the balance of plasticity toward burst-dependent LTP in DA neurons and may contribute to the development of drug addiction.

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CHAPTER 1: INTRODUCTION

Drug addiction is a serious public health and social problem that impacts our society on multiple levels. Addiction-related economical cost, including health care expenditures, lost earnings, and costs associated with drug-related crime and accidents, exceeds 484 billion dollars each year in the United States (public data on NIDA website, http://www.nida.nih.gov/about/welcome/aboutdrugabuse/magnitude/).

A defining characteristic of drug addiction is the transition from initial entertainment drug use into a compulsive state where drug use is out of control in spite of numerous adverse health, social, and legal consequences (Koob & Le Moal 2005). Accumulating evidence over the past decade suggests that this transition is associated with long-lasting changes in the synaptic transmission onto dopamine (DA) neurons in the ventral midbrain (Jones & Bonci 2005; Kauer 2004; Vezina 2004). Repeated exposure to addictive drugs produces potentiation of glutamatergic synapses in DA neurons (Borgland et al 2004; Liu et al 2005; Saal et al 2003). Since intracellular Ca^{2+} ($[Ca^{2+}]_i$) is critically involved in controlling synaptic plasticity (Berridge 1998; Lisman 1989), delineating cellular mechanisms that regulate Ca^{2+} signaling in DA neurons may provide a new avenue for us to understand the neuronal adaptations underlying the development of drug addiction. In this dissertation, we discovered a unique

mechanism, by which two distinct Ca^{2+} signals were differentially regulated in DA neurons. These two types of Ca^{2+} signals -- action potential (AP)-induced Ca^{2+} transient and metabotropic glutamate receptor (mGluR)-mediated Ca^{2+} wave, have been associated with long-term potentiation (LTP) (Liu et al 2005; Pu et al 2006) and long-term depression (LTD) of glutamate transmission (Bellone & Luscher 2005; 2006), respectively in DA neurons. Our results demonstrated that an increase in the intracellular inositol 1,4,5-trisphosphate (IP_3) tone resulting from activation of metabotropic receptors-coupled to phospholipase C (PLC)-mediated phosphoinositide (PI) hydrolysis, such as mGluRs, α1 adrenergic receptors (α1ARs), and muscarinic acetylcholine receptors (mAChRs), augmented AP-induced Ca^{2+} signals while inhibiting mGluR-induced Ca^{2+} responses. Our findings suggest a novel mechanism in which these neurotransmitter systems may affect the function of DA neurons and contribute to reward learning and drug addiction.

This introduction summarizes current views on DA neuron function, drug addiction, and neuronal Ca^{2+} signaling.

1.1 DA theory of reward learning and drug addiction

Reward-based reinforcement learning, which associates certain cues or actions (e.g. pressing a lever in a behavioral test) with a rewarding outcome (procurement of food, water, sex, etc.), is essential for human beings and animals to survive and propagate their species. On the other hand, the neural substrates mediating this learning are also targets for drugs of abuse (Wise 1998). Repeated exposure to addictive drugs may cause adaptive changes in these brain structures and result in a sustained addictive state (Hyman et al 2006; Redish 2004; Robinson & Berridge 2001).

1.1.1 BRAIN REWARD CIRCUITRY

Our current knowledge about brain reward circuitry was established on the original findings by Olds and Milner, who found that rats would return to the place where they received electrical stimulation in the septal area of the brain, and could be trained to press a lever for such stimulation (Olds 1958; Olds & Milner 1954). Such findings of presumed 'reward center' in the brain encouraged subsequent mapping studies using both intracranial self-stimulation and self-administration techniques. A variety of disparate brain structures have since then been identified as potential components of the brain reward circuitry (Ikemoto & Wise 2004; Phillips 1984), with the most sensitive sites along the medial forebrain bundle (MFB) (Wise 1996) which runs between ventral

tegmentum and lateral hypothalamus. Among many of the these structures, the dopaminergic projection systems have thus far received most attention, because early studies showed that DA systems were crucial for reward learning and drug addiction. For example, DA receptor antagonists attenuated intracranial self stimulation of MFB at doses that did not induce motor deficits (Fouriezos & Wise 1976; Franklin & McCoy 1979; Zarevics & Setler 1979). Lesion of DA systems with 6-hydroxydopamine (6-OHDA) or blocking DA receptors with antagonists inhibited animal's drive for natural rewards (Gerber et al 1981; Ungerstedt 1971; Wise et al 1978). Most importantly, DA antagonists blocked the reinforcing effect of additive drugs, such as amphetamine and cocaine, suggesting a essential role of DA systems in drug addiction (De Wit & Wise 1977; Yokel & Wise 1975).

DA neuron cell bodies are located in the ventral midbrain and grouped into two major nuclei: the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). SNc DA neurons project to the dorsal striatum, while VTA DA neurons project to limbic structures such as nucleus accumbens, prefrontal cortex, cingulate cortex, and hippocampus (Gasbarri et al 1994; Oades & Halliday 1987; Scatton et al 1980). These two projection pathways, termed nigrostriatal DA system and mesolimbocortical DA system, respectively, are not clearly bordered and have some overlap in their projections (Fallon & Moore 1978). Both of these pathways have been implicated in motor and reward-related behaviors, although the mesolimbocortical pathway is thought to be more important for reward and motivation (Wise 2004).

1.1.2 HYPOTHESES OF DA FUNCTION IN REWARD LEARNING AND DRUG ADDICTION

Although there is substantial evidence suggesting that DA systems are critically involved in reward learning and drug addiction, what precisely DA does during these processes is still a highly debatable issue (Berridge 2006; Wise 2004). Current views can be generally categorized into three hypotheses: the hedonia hypothesis, the incentive salience hypothesis, and the reward prediction error hypothesis.

1.1.2.1 The hedonia hypothesis

The hedonia hypothesis posits that DA mediates the subjective feeling of pleasure or euphoria associated with rewarding stimuli (Wise 1980). It was developed by Wise in 1970s and 1980s, based on the results that blockade or lesion of DA systems attenuated intracranial self-stimulation (Fouriezos & Wise 1976; Franklin & McCoy 1979; Zarevics & Setler 1979), and inhibited the reinforcing effect of natural rewards (Gerber et al 1981; Ungerstedt 1971; Wise et al 1978). It is also supported by more recent studies showing that DA systems are activated by almost all natural and social rewards (Becker et al 2001; Roitman et al 2004; Thut et al 1997). More importantly, human brain imaging studies showed that DA release correlated with feeling of pleasure induced by palatable food (Small et al 2003) and by psychostimulants methylphenidate (Volkow et al 1999) and amphetamine (Drevets et al 2001).

However, this hypothesis has been challenged on various grounds. For example, DA neurons are not only activated by reward-related stimuli, they can

also be activated by novel, incentive, non-rewarding stimuli that usually require animals to make an immediate response (Ljungberg et al 1992; Schultz & Romo 1990). The hedonia hypothesis can not explain why animals can be trained to work for noxious stimuli (Kelleher & Morse 1968), and why some people will compulsively commit self-poisoning and self-injury (Horrocks & House 2002). Furthermore, drug-addicted patients would continue to seek drugs despite the rapid decrease in the drug-induced subjective feeling of euphoria (Foltin & Fischman 1991; Lamb et al 1991; Russell 1989).

1.1.2.2 The incentive salience hypothesis

Another challenge to the hedonia hypothesis came from studies initiated by Berridge and Robinson (Berridge 2000; Robinson & Berridge 1993), who argued that hedonic impact of a reward is not purely a subjective feeling, but also has an objective side (Berridge 2006), and thus can be directly measured in animal behaviors. They used sweet or bitter taste to induce stereotypical affective facial expressions in rodents (which look similar to the reaction in human infants), and used these responses to quantify hedonia and to discriminate 'liking' (hedonic impact) from 'wanting' (the motivation to pursue the reward) (Steiner et al 2001). They found that after 6-OHDA lesion of DA systems (Berridge & Robinson 1998; Berridge et al 1989) or local injection of DA receptor antagonist (Pecina et al 1997), rats still displayed the 'liking' responses to sucrose.

To reconcile the discrepancy between their findings (DA was not a liking signal) and previously established role of DA in reward-related learning, Robinson and Berridge formulated the incentive salience hypothesis of DA, which is based on an assumption that a reward is composed of three dissociable elements: liking, learning, and wanting. The central message of the incentive salience hypothesis is that "Dopamine mediates only a 'wanting' component, by mediating the dynamic attribution of incentive salience to reward-related stimuli, causing them and their associated reward to become motivationally 'wanted'" (Berridge 2006). Thus disruption of DA systems only affects animals' wanting but not their liking of the reward.

The incentive salience hypothesis received supports from behavioral studies using DA deficient (DD) mice (Cannon & Palmiter 2003; Hnasko et al 2005; Robinson et al 2005). DD mice lack tyrosine hydroxylase and can not synthesize DA precursor L-dopa. These mice display severe DA deficit symptoms such as akinesia, aphasia, adipsia (Zhou & Palmiter 1995), and thus have to receive daily injection of L-dopa to maintain their normal level of eating and drinking behaviors. Cannon and Palmiter found that although DD mice did not voluntarily drink enough to maintain their body need without medication, they did sometimes drink. And when they did so, they showed profound preference for sucrose over water, just as in wild type mice (Cannon & Palmiter 2003), but see (Martinez-Hernandez et al 2006). Furthermore, they found that morphine-induced conditioned place preference (CPP) was not affected in DD mice

(Hnasko et al 2005). The authors concluded that DA was not required for the hedonic effect of rewards (liking), nor was it necessary for reward learning. It only mediated the motivational 'wanting' of the rewards (Cannon & Bseikri 2004; Cannon & Palmiter 2003; Hnasko et al 2005; Robinson et al 2005). It should be noted however, that the information obtained from DD mice does not necessarily rule out the function of DA in reward learning in normal animals, since it is quite common that normal functions of a particular gene could be taken over by other compensatory mechanisms in gene-knockout mice.

1.1.2.3 The reward prediction error hypothesis

A currently more influential hypothesis about the DA function is the reward prediction error hypothesis (Schultz 1998; 2002). Schultz and colleagues developed this hypothesis based on their recordings of DA neuron spike activities in awake behaving monkeys during classical and operant conditioning experiments. In resting conditions, DA neurons mostly fire single spikes at relatively low frequencies. When the monkey is presented with an unexpected primary reward (e.g. food, sweet juice), DA neurons respond by switching from tonic firing to burst firing (Hollerman & Schultz 1998; Schultz et al 1993). If a neutral cue (e.g. a flash of light, or a tone) is repeatedly presented shortly before the delivery of reward, DA neurons start to respond to the cue that precedes the reward, and the response to the actual reward gradually weakens (Hollerman & Schultz 1998; Ljungberg et al 1992; Mirenowicz & Schultz 1994). After the animal learns that the cue is a reliable predicator of reward, DA neurons only

burst at the cue and stop responding to the actual reward (Hollerman & Schultz 1998). If the reward is omitted at the expected delivery time, DA neurons will respond with a decrease in firing rate (Ljungberg et al 1991; Mirenowicz & Schultz 1994; Schultz et al 1993). These results indicate that DA neurons do not signal the reward per se, but the reward prediction error. Thus, if the actual reward comes as expected (error = 0), DA neurons will not change their activities. If the reward is unpredicted or better than predicted (error > 0), DA neuron will respond with a burst of firing. If the actual outcome is worse than expected (error < 0), DA neurons will cease firing or decrease their firing rate.

The prediction error hypothesis fits well into computational reinforcement-learning models (Montague et al 2004; Sutton 1998). These models are based on the assumption that animals always act to maximize future rewards. During reward learning, brain associates certain reward values with the animal's actions. Before making a behavioral response, animals will estimate the reward value of the next action according to the stored values of past actions. If the actual outcome of next action is better than the originally stored value, that is, the prediction error is positive, this action will be reinforced. Otherwise, the action will be aborted or avoided in the future. DA is believed to serve as the prediction error signal during this process.

These models have been used to explain why addictive drugs can beat all other rewards and become the sole goal of addicted people's lives (Montague et al 2004; Redish 2004). Since all addictive drugs stimulate DA release (see

section 1.1.4.1) by their direct pharmacological actions, every time when the drug is taken, the brain will interpret it as a better reward than predicted, regardless of the actual subjective feelings. Thus the drug-induced pharmacological release of DA serves as a 'fake' positive reward prediction error signal, which 'fools' the brain to reinforce the drug-taking behaviors. These 'fake' signals cause pathological overlearning of drug-related cues and behaviors, and at the same time devalue all other rewards, resulting in a compulsive drug-seeking state.

In summary, the hedonia hypothesis can very well explain the initiation of drug addiction, because drug-induced hedonic impact is after all the very reason that attracts people to start using drugs, whereas reward-prediction error and incentive salience hypotheses have more advantages in explaining the transition to the addicted state when hedonic feeling and drug use are dissociated.

1.1.3 BASIC PROPERTIES OF DA NEURONS

The extracellular DA level is controlled by firing activity of DA neurons (Garris et al 1994; Gonon 1988; Manley et al 1992). DA neurons fire APs in two distinct patterns *in vivo*: tonic, single-spike firing (1-10 Hz) and phasic, burst firing, which is often followed by a pause of activity (Freeman & Bunney 1987; Kitai et al 1999; Overton & Clark 1997). It is believed that DA neuron bursts, giving rise to phasic DA signals in projection areas, provide the learning signal,

while tonic levels of DA, maintained by tonic DA neuron firing, act to facilitate, or motivate, already learned behaviors (Cagniard et al 2006; Floresco et al 2003).

1.1.3.1 Single-spike firing of DA neurons

DA neurons are spontaneously active in vivo and in vitro (Fujimura & Matsuda 1989; Grace & Bunney 1984b). Excitatory synaptic input is not required to maintain the tonic firing, although the basal firing rate are affected by both excitatory and inhibitory inputs (Adell & Artigas 2004). DA neurons display regular slow pacemaker firing pattern in brain slices (Grace & Bunney 1983; Morikawa et al 2003). Na^+ channel blocker tetrodotoxin (TTX) eliminates AP spikes and reveals a slow autonomous membrane potential oscillation (Fujimura & Matsuda 1989; Grace & Onn 1989; Nedergaard et al 1993). This oscillation is blocked by selective L-type voltage-gated Ca^{2+} channel (VGCC) blockers (Harris et al 1989; Mercuri et al 1994; Nedergaard et al 1993), suggesting an essential role of L-type channels in the slow depolarizing phase during the oscillation. Indeed, DA neurons express high level of Cav 1.3 Ca^{2+} channels in the soma and dendrites (Takada et al 2001), which start to open at around -50 mV and have a slow inactivation time constant (Olson et al 2005; Xu & Lipscombe 2001). The hyperpolarizing phase of the membrane potential oscillation is sensitive to intracellular Ca^{2+} chelators and small conductance Ca^{2+}-sensitive K^+ (SK) channel blocker apamin (Grace & Bunney 1984b; Ping & Shepard 1996). Thus, it has been proposed that the intrinsic membrane potential oscillation underlying the pacemaker firing of DA neurons is mainly caused by Ca^{2+} influx through low

threshold L-type VGCCs and subsequent SK channel activation (Wilson & Callaway 2000). However, there is other evidence suggesting that the major VGCCs coupled to SK channel activation in DA neurons are T-type (Cui et al 2004; Wolfart & Roeper 2002).

1.1.3.2 Burst firing of DA neurons:

DA neurons switch their firing pattern from basal single-spike firing to burst firing in response to unexpected rewards and reward-predicting stimuli (Schultz 2002). Bursts have two significant advantages over single-spike firing in transmitting information: they are more efficient in increasing extracellular DA concentration at target areas (Gonon 1988; Suaud-Chagny et al 1992), and at dendrites, postsynaptic bursts are known to produce activity-dependent plastic changes, mainly via causing profound membrane depolarization, which produces supralinear $[Ca]_i$ rise by activating VGCCs or by removing Mg^{2+} blockade to facilitate Ca^{2+} influx through NMDA receptors (NMDARs) (Dan & Poo 2006).

In contrast to tonic single-spike firing, burst firing in DA neurons is afferent-dependent. Bursts occur much more frequently in freely moving animals (Freeman & Bunney 1987; Miller et al 1983) than in anaesthetized or paralyzed ones (Bunney et al 1973; Grenhoff et al 1988; Schultz & Romo 1987). They are absent in brain slices unless proper stimulation is applied (Grace & Onn 1989; Morikawa et al 2003). Electrical stimulation of glutamatergic fibers or direct iontophoretic application of glutamate induces bursts in DA neurons in vivo (Chergui et al 1994; Floresco et al 2003; Grace & Bunney 1984a) and in vitro

(Morikawa et al 2003). Locally blocking NMDARs but not AMPA receptors (AMPARs) eliminates burst firing (Charlety et al 1991; Chergui et al 1993; Morikawa et al 2003; Overton & Clark 1992), suggesting a necessary role of NMDAR activation in burst firing of DA neurons.

DA neurons receive glutamatergic afferents from prefrontal cortex (PFC) (Beckstead 1979; Carter 1982; Christie et al 1985; Sesack & Pickel 1992), subthalamus nucleus (Kita & Kitai 1987; Smith & Grace 1992), amygdala (Gonzales & Chesselet 1990; Phillipson 1979; Wallace et al 1989), pedunculopontine tegmental nucleus (PPTg) (Futami et al 1995; Jackson & Crossman 1983), laterodorsal tegmental nucleus (LDTg) (Semba & Fibiger 1992), and the bed nucleus of the stria terminalis (Georges & Aston-Jones 2001; Phillipson 1979). PFC has been suggested to be the major driving force for DA neuron bursts, because inactivation of PFC by cooling (Svensson & Tung 1989) or local injection of anesthetics (Murase et al 1993) suppresses burst firing without affecting the basal firing rate. Electrical stimulation of PFC has been shown to induce burst firing in DA neurons (Gariano & Groves 1988; Tong et al 1995). However, recent studies also suggested necessary roles of glutamate afferents from PPTg and LDTg for DA neuron burst firing. Blocking PPTg input depressed reward predicting cue-induced burst firing in DA neurons (Pan & Hyland 2005). Inactivating LDTg not only eliminated bursts in DA neurons and but also blocked the burst induced by direct iontophoretic application of glutamate onto DA neurons (Lodge & Grace 2006).

In addition to glutamatergic innervation, DA neurons are also under the impact of multiple neurotransmitter systems, such as GABA-ergic, cholinergic, and adrenergic systems (Kitai et al 1999; Paladini & Williams 2004). All of these systems have been implicated in modulating DA system function during reward-based learning and drug addiction. Repeated drug exposure may produce plastic changes in both intrinsic properties of DA neurons and afferent regulation of these systems.

1.1.4 NEUROADAPTIVE CHANGES OF DA SYSTEMS DURING DRUG ADDICTION

1.1.4.1 Acute pharmacological effects of addictive drugs on DA systems

As mentioned previously, stimulation of DA systems is a common feature shared by all additive drugs (Koob 1992). It constitutes the initial step that triggers a series of adaptive changes in DA systems leading to the final addictive state. However, the action sites and mechanisms vary among different types of drugs.

Psychostimulants such as cocaine and amphetamine increase extracellular monoamine (DA, norepinephrine [NE], and serotonin [5-HT]) concentrations by acting on monoamine transporters (DA transporter [DAT], NE transporter [NET], and 5-HT transporter [SERT]). Cocaine increases extracellular monoamine concentrations by blocking monoamine transporter thus inhibiting monoamine reuptake (Ritz et al 1987), whereas amphetamine releases

monoamines by binding to their transporters as a false substrate thereby causing reverse transport of cytosolic transmitters into extracellular space (Seiden et al 1993).

Results from behavioral studies suggest that the primary action sites mediating the reinforcing effect of cocaine and amphetamine may be different. Rats readily self-administer amphetamine into NAc (Hoebel et al 1983; Phillips et al 1994), orbitofrontal cortex (Phillips et al 1981), and central nucleus of amygdala (Chevrette et al 2002). This effect requires activation of both D1- and D2- DA receptors. Blocking either one of them diminishes self-administration (Phillips et al 1994). Rats only self-administer the combination of D1- and D2-agonists, but not either one alone (Ikemoto et al 1997). Although cocaine administration also elevates DA concentration in NAc (Hurd et al 1988), unlike amphetamine, intra NAc self-administration of cocaine seems to be much more difficult than intra-PFC and intra-olfactory tubercle self-administration (Goeders & Smith 1983; Ikemoto 2003), suggesting that PFC and olfactory tubercle may play more important roles in mediating the reinforcing effect of cocaine.

Opiates such as heroin and morphine have both DA-dependent effects in the VTA and DA-independent effects in the NAc. They are self-administered into the VTA (Bozarth & Wise 1980), and intra-VTA administration of opiates induces CPP (Bozarth 1987). In the VTA, opiates activate μ-opioid receptors on local GABA-ergic interneurons thus stimulating DA neurons via disinhibition (Johnson & North 1992). In the NAc, the major effect of opiates is to suppress

glutamatergic synapses on GABA-ergic medium spiny neurons by activating presynaptic μ- and δ- receptors (Jiang & North 1992).

Ethanol modulates the functions of a variety of ion channels at clinically relevant concentrations. For example, It enhances the activation of $GABA_A$ and glycine receptors (Allan & Harris 1986; Mihic et al 1997), activates G-protein-activated inward rectifier K^+ channels (Kobayashi et al 1999), and inhibits NMDARs (Peoples & Weight 1995) and certain types of voltage-gated K^+ channels (Covarrubias & Rubin 1993). At system level, activation of DA pathways has long been implicated in ethanol reinforcement (Samson et al 1990; Weiss et al 1993). Ethanol is self-administered into VTA (Gatto et al 1994; Rodd-Henricks et al 2000), and causes excitation of DA neurons (Gessa et al 1985). It has been recently demonstrated that ethanol directly stimulates DA neuron firing by inhibiting K^+ conductances (Brodie et al 1999) and by enhancing I_h currents (Okamoto et al 2006).

Cannabinoid Δ^9 tetrahydrocannabinol (Δ^9THC), the active component in marijuana, is self-administered intravenously (Tanda et al 2000), produces CPP (Valjent & Maldonado 2000), and enhances intracranial self-stimulation (Gardner et al 1988). These reinforcing effects correlate with potentiated DA transmission in NAc, and are thought to be mediated by CB1 receptor-induced presynaptic inhibition of GABA-ergic synapses in VTA and NAc (Lupica et al 2004). This hypothesis is supported by a recent behavioral study showing that co-

administration of CB1 receptor antagonist rimonabant blocks self-administration of Δ^9THC into VTA and NAc (Zangen et al 2006).

Taken together, the acute reinforcing effect of all kinds of addictive drugs is closely associated with activation of DA projection pathways. However, this acute effect itself does not explain the shift from initial volitional drug-taking to drug addictive state. It is now believed that this behavioral change is related to enduring adaptive changes in DA systems caused by repeated drug exposure, including enhanced glutamatergic transmission in DA neurons (Kauer 2004; Vanderschuren & Kalivas 2000; Wolf 1998).

1.1.4.2 Repeated drug exposure-induced plastic changes in DA systems

1.1.4.2.1 Behavioral sensitization

Repeated administration of addictive drugs causes progressive and enduring enhancement of locomotor stimulating effects in rodents (Kalivas & Stewart 1991). This phenomenon, termed behavioral sensitization, has been extensively studied as an animal model of drug-craving behavior in human beings (Robinson & Berridge 1993; Vanderschuren & Kalivas 2000), because 1) reinforcing and locomotor-stimulating effects of addictive drugs share common neural pathways (Wise & Bozarth 1987); 2) locomotor sensitization is associated with augmented drug-induced DA release (Kalivas & Duffy 1993; Wolf et al

1993); 3) locomotor-sensitized animals display enhanced drug self-administration and drug-seeking behavior (Ferrario & Robinson 2006; Valadez & Schenk 1994; Vezina 2004; Vezina et al 2002); and 4) behavioral sensitization is extremely long-lasting (Robinson & Berridge 1993), which is comparable to the persistence of drug-craving in humans. Behavioral sensitization is best characterized in psychostimulants, but can also be induced by opiates and ethanol (Phillips et al 1997; Vanderschuren & Kalivas 2000).

1.1.4.2.2 Necessary role of VTA glutamate afferents in the induction of behavioral sensitization

Behavioral sensitization can be induced by intra-VTA administration of cocaine (Cornish & Kalivas 2001), amphetamine (Hooks et al 1992; Kalivas & Weber 1988), and morphine (Vezina et al 1987), whereas repeated intra-NAc (Dougherty & Ellinwood 1981; Kalivas & Weber 1988) and intra-PFC (Hooks et al 1992) injections fail to do so. These results indicate that the induction of behavioral sensitization, which models the critical period of transition from initial drug use to addictive state, is mainly mediated by neuroadaptive changes in the VTA.

Since VTA glutamatergic afferents control burst activity of DA neurons (see section 1.1.3.2), and burst firing is more efficient than single-spike firing in causing DA release (Gonon 1988), it is highly possible that behavioral sensitization and associated augmentation in drug-induced DA release are mediated by enhanced glutamate transmission onto DA neurons. This idea is

supported by studies showing that amphetamine, cocaine, and morphine-induced locomotor sensitization is blocked by intra-VTA administration of NMDAR (Cador et al 1999; Kalivas & Alesdatter 1993; Vezina & Queen 2000), AMPAR (Licata et al 2004), and mGluR (Kim & Vezina 1998) antagonists, indicating a necessary role of glutamate afferents for induction of behavioral sensitization. Furthermore, after repeated systemic cocaine and amphetamine administration, the responsiveness of DA neurons to iontophoretic application of glutamate is significantly enhanced (White et al 1995; Zhang et al 1997). Burst firing induced by electrical stimulation of prefrontal cortex has also been shown increased after repeated drug exposure (Tong et al 1995). Among various sources of glutamate afferents on to DA neurons (see section 1.1.2.2), the PFC input seem to be the major afferent that mediate the sensitization, because lesions of medial PFC prevent the development of cocaine-, amphetamine-, and morphine-induced locomotor sensitization (Cador et al 1999; Tzschentke & Schmidt 1999; Wolf et al 1995), whereas electrical stimulation of PFC can mimic repeated drug exposure and induce behavioral sensitization to subsequent systemic cocaine administration (Schenk & Snow 1994).

The critical role of VTA glutamate afferents in drug addiction also receives evidential support from other behavioral models. For example, intra-VTA blockade of glutamate receptors eliminates cocaine-induced CPP (Harris & Aston-Jones 2003; Kim et al 2004). Blocking glutamate transmission with

kynurenate, or blocking NMDARs with AP-5 or ketamine in the VTA attenuates heroin and cocaine self-administration (Sun et al 2005; Xi & Stein 2002).

Taken together, these results suggest that glutamatergic synapses in VTA constitute the best candidate for initial neural adaptations during the development of drug addiction.

1.1.4.2.3 Synaptic plasticity in DA neurons

Synaptic plasticity has been proposed to mediate many experience-dependent neural adaptations including learning and memory (Martin et al 2000). Both LTP and LTD have been elicited at glutamatergic synapses in DA neurons in brain slices by several groups using different induction protocols (Bellone & Luscher 2005; Gutlerner et al 2002; Liu et al 2005; Ungless et al 2001). Most of the addictive drugs have been shown to increase the efficacy of glutamatergic transmission in VTA DA cells, either by disinhibition mechanisms or by promoting LTP-like potentiation. For example, superfusion of amphetamine or DA blocks DA neuron LTD (Jones et al 2000; Thomas et al 2000). Repeated or a single administration of cocaine in vivo induces LTP in DA cells in vitro (Liu et al 2005; Saal et al 2003; Ungless et al 2001). Furthermore, amphetamine, morphine, ethanol and nicotine all promote LTP-like potentiation of AMPAR-mediated synaptic responses in VTA DA neurons (Saal et al 2003). More importantly, the magnitude of cocaine-induced LTP in DA neurons correlates with the magnitude of cocaine-induced locomotor stimulating effects (Borgland et al 2004). All together, these data suggest that addictive drugs share a common feature of

strengthening the synaptic connections between glutamatergic afferents and DA neurons.

1.1.4.2.4 Plasiticity in other reward-related brain regions

LTP and LTD have also been observed in glutamatergic synapses on GABA-ergic medium spiny neurons (MSNs) in the NAc (Kombian & Malenka 1994; Thomas et al 2000). Repeated cocaine in vivo produces LTD in MSN synapses (Thomas et al 2001), whereas strengthening these synapses by over-expression of GluR1 expedites the extinction of cocaine-seeking behaviors, and also makes cocaine aversive rather than rewarding to animals (Kelz et al 1999; Sutton et al 2003). These data suggest that LTD in MSN synapses is related to enhanced motivational and reinforcing effects of addictive drugs. Indeed, blocking LTD in these synapses prevents amphetamine-induced behavioral sensitization (Brebner et al 2005). It has also been shown that DA and addictive drugs can modulate synaptic plasticity in PFC (Gurden et al 1999; Huang et al 2004; Otani et al 1998), amygdala (Bissiere et al 2003), hippocampus (Huang & Kandel 1995; Pu et al 2002; Roberto et al 2002), and BNST (Dumont et al 2005), but the functional relationship of plasticity changes in these areas with drug addiction is still less clear than that in the VTA and NAc.

In summary, we now have good evidence that drug administration induces long-term plastic changes in VTA and other structures in brain reward circuitry. The question that remains obscure is how additive drugs produce these plastic

changes. As in all other central synapses, synaptic plasticity in VTA is Ca^{2+}-dependent (Bellone & Luscher 2005; Jones & Bonci 2005; Jones et al 2000; Thomas et al 2000). The magnitude and direction of synaptic plasticity are believed to be determined by the spatiotemporal profile of $[Ca^{2+}]_i$ (Lisman 1989). It is thus logical to hypothesize that addictive drug-induced potentiation in DA neurons may be related to altered Ca^{2+} signaling under the impact of these drugs. The next section will be focused on neuronal calcium signaling mechanisms in DA neurons and how they are regulated by addictive drugs.

1.2 Regulation of Ca^{2+} signaling in DA Neurons

1.2.1 NEURONAL CA^{2+} SIGNALING PATHWAYS

Ca^{2+} plays multiple roles in controlling the neuronal activities, ranging from affecting the membrane excitability to regulating the synaptic plasticity and gene transcription (Berridge 1998). $[Ca^{2+}]_i$ can be elevated either via Ca^{2+} influx from extracellular space through Ca^{2+} permeable channels, or via Ca^{2+} mobilization from intracellular stores (Fig. 1.1). The major Ca^{2+} channels on the plasma membrane include VGCCs and ligand-gated Ca^{2+} permeable channels such as NMDARs and certain types of AMPARs (Hellie 2001). The main Ca^{2+} store inside the cell is the endoplasmic reticulum (ER) (Henkart 1980; Henkart et al 1978; Verkhratsky 2005), which is a continuous network distributed throughout the cell and even follows the cell membrane into dendritic spines and nerve terminals (Spacek & Harris 1997; Westrum & Gray 1986). Two types of intracellular Ca^{2+} channels are expressed on ER membrane: IP_3 receptors (IP_3Rs) (Berridge 1993; Mignery et al 1989) and ryanodine receptors (RyRs) (Berridge 1993). Both of these two channels can be co-activated by Ca^{2+} and the corresponding Ca^{2+} mobilizing messengers: IP_3 for IP_3Rs (Bezprozvanny et al 1991; Finch et al 1991) and cyclic ADP ribose (cADPR) for RyRs (Galione et al 1991; Meszaros et al 1993).

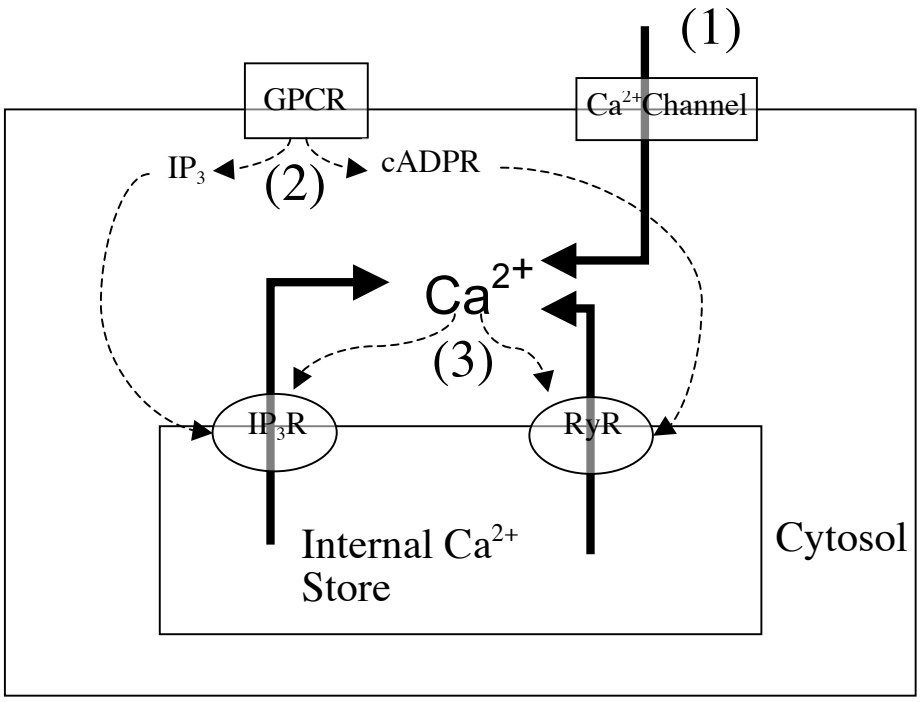

Figure 1.1 Neuronal Ca^{2+} signaling pathways.

Three major pathways are illustrated: (1) Ca^{2+} influx through Ca^{2+} permeable channels on the plasma membrane; (2) messenger-induced Ca^{2+} release from intracellular stores following activation of PI-coupled metabotropic receptors (GPCR); and (3) CICR through IP_3Rs and RyRs.

1.2.1.1 Ca^{2+}-induced Ca^{2+} release (CICR)

Ca^{2+} influx through Ca^{2+} channels on the plasma membrane can activate IP_3Rs (Dyachok & Gylfe 2004; Miyazaki et al 1992; Roderick et al 2003) and RyRs (Friel & Tsien 1992), and release Ca^{2+} from intracellular stores (Berridge 1998; Verkhratsky & Shmigol 1996). One typical example of physiological CICR is AP-induced Ca^{2+} signaling, in which APs activate VGCCs, and the Ca^{2+} influx through VGCCs is then amplified by Ca^{2+} release from intracellular stores (Cohen et al 1997; Emptage et al 2001; Hillsley et al 2000; Kato et al 1999; Sandler & Barbara 1999). The consequent $[Ca^{2+}]_i$ rise often couples to Ca^{2+}-sensitive K^+ channels and induces a large post-spike hyperpolarization, termed afterhyperpolarization (AHP) (Hillsley et al 2000; Moore et al 1998), which plays a critical role in controlling the neuronal excitability (Bond et al 1999). CICR has also been implicated in synaptic plasticity. Depleting intracellular Ca^{2+} stores or blocking RyRs with dantrolene inhibits LTP in hippocampal CA1 neurons (Harvey & Collingridge 1992; Obenaus et al 1989). Both AP- and synaptic NMDAR-induced $[Ca^{2+}]_i$ rise in hippocampal dendritic spines are remarkably suppressed by blocking CICR (Emptage et al 1999; Emptage et al 2001)

1.2.1.2 Messenger-mediated Ca^{2+} mobilization from intracellular stores

IP_3 and cADPR are two major identified Ca^{2+} releasing messengers (Berridge 1993; Higashida et al 2001). IP_3 is produced following activation of metabotropic receptors-coupled to PLC-mediated PI hydrolysis, such as mGluRs, α1ARs, and mAChRs (Berridge 1993). Depending on the input intensities and

cell types, various forms of $[Ca^{2+}]_i$ responses can be induced by activation of PI-coupled receptors. Highly localized IP_3-mediated Ca^{2+} transients were evoked by synaptic stimulation of mGluRs in cerebellar Purkinje cells, where $[Ca^{2+}]_i$ increase was confined within the dendritic spines (Finch & Augustine 1998). This focal Ca^{2+} signaling is necessary and sufficient to induce LTD in nearby synapses (Svoboda & Mainen 1999). Propagating Ca^{2+} wave can be elicited by synaptic stimulation of mGluRs and mAChRs in hippocampal pyramidal neurons (Jaffe & Brown 1994; Nakamura et al 1999), by stimulation of mGluRs in neocortical pyramidal neurons (Larkum et al 2003), and by stimulation of mGluRs, mAChRs, and α1ARs activation in midbrain DA neurons (Fiorillo & Williams 2000; Morikawa et al 2003; Paladini & Williams 2004). This propagating Ca^{2+} signal has been proposed to transfer information into the nucleus and trigger gene transcription in response to extracellular stimuli (Berridge 1998; Nakamura et al 1999). In addition to directly causing Ca^{2+} release, low concentration of IP3 produced by weak stimulation of mGluRs, which by itself does not induce Ca^{2+} release, can facilitate the CICR induced by back propagating APs in hippocampal pyramidal cells (Nakamura et al 1999).

cADPR is another emerging Ca^{2+} mobilizing messenger that acts on RyRs (Galione et al 1991; Higashida et al 2001; Meszaros et al 1993). Recent evidence suggests that production of cADPR is also coupled to mAChRs, mGluRs, and α1ARs in cultured neuroblastoma cells and in DA neurons (Higashida et al 1997; Morikawa et al 2003; Paladini & Williams 2004). cADPR

can either directly activate RyRs and induce Ca^{2+} release (Meszaros et al 1993; Morikawa et al 2003), or act as a co-activator and assist CICR (Hua et al 1994).

1.2.2 $[Ca^{2+}]_i$ AND DA NEURON EXCITIAILITY

The major function of $[Ca^{2+}]_i$ in regulating neuronal excitability is to produce hyperpolarizations via activating Ca^{2+}-sensitive K^+ channels, such as large conductance (BK) (Marty 1981) and small conductance (SK) Ca^{2+}-sensitive K^+ channels (Blatz & Magleby 1986), whose roles are best characterized in the generation of AHPs (Sah & Faber 2002). Depending on cell types, three temporally and pharmacologically distinctive AHPs have been isolated. Fast AHP, which starts immediately during the action potential and decays within several tens of milliseconds, is mediated by BK channels (Adams et al 1982; Shao et al 1999). Medium AHP (mAHP, or AHP), which lasts for several hundred milliseconds, is dependent on activation of SK channels (Sah 1992; Sah & McLachlan 1991; 1992). In some neurons a slow AHP (sAHP), which is Ca^{2+}-depedent and lasts up to several seconds has also been seen (Hirst et al 1985; Sah 1996), but the channels mediating sAHP and the intracellular mechanisms are still not clear. A recent study suggests that hippocalcin serves as an intracellular Ca^{2+} sensor that gates the Ca^{2+}-sensitive K^+ channels mediating sAHP in hippocampal pyramidal cells (Tzingounis et al 2007).

In DA neurons, rhythmic membrane potential oscillations caused by VGCC-mediated Ca^{2+} influx and subsequent activation of SK channels constitute

the major driving force for intrinsic firing (Wilson & Callaway 2000). Ca^{2+} influx through nifedipine-sensitive L-type and ω-Aga-IVA-sensitive P/Q type Ca^{2+} channels depolarizes the membrane to reach the threshold of Na^{2+} channels and induces an AP (Mercuri et al 1994; Puopolo et al 2007). On the other hand, APs selectively activate low threshold T-type VGCCs (Wolfart & Roeper 2002). The consequent Ca^{2+} influx, further amplified by CICR, activates SK channels, leading to the generation of large AHPs (Wolfart et al 2001; Wolfart & Roeper 2002).

Furthermore, transient activation of mGluRs, which produces IP_3 and cADPR activating IP_3Rs and RyRs, respectively, triggers massive Ca^{2+} release from intracellular stores, causing a prolonged SK-mediated hyperpolarization that underlies a pause of activity curtailing phasic bursts driven by ionotropic glutamate receptors (iGluRs) (Fiorillo & Williams 1998; Morikawa et al 2003). Similar SK-mediated inhibition is also seen induced by transient activation of mAChRs and α1ARs in DA neurons (Fiorillo & Williams 2000; Paladini & Williams 2004)

In addiction to activating Ca^{2+} sensitive K^+ channels, it has also been shown that activation of metabotropic receptors depolarizes the membrane and induces Ca^{2+} influx in hippocampal CA3 pyramidal cells (Gee et al 2003), DA cells (Tozzi et al 2003), and neocortical neurons (Prothero et al 2000) via activating non-selective cationic channels.

1.2.3 $[Ca^{2+}]_i$ AND DA NEURON SYNAPTIC PLASTICITY

1.2.3.1 Functional specificity of $[Ca^{2+}]_i$ in controlling synaptic plasticity

Synaptic plasticity is thus far the best cellular model underlying learning and memory (Malenka & Bear 2004). It is a highly dynamic process so that the same synapses can switch between LTP and LTD in response to appropriate stimulation (Heynen et al 1996). It is generally accepted that the bidirectionality of changes in synaptic plasticity is controlled by the magnitude and spatiotemporal profile of $[Ca^{2+}]_i$ rise resultinf from varying Ca^{2+} sources and different forms of stimuli (Cummings et al 1996; Linden 1994; Linden & Connor 1995; Lisman 1989). Ca^{2+} signals associated with postsynaptic APs and activation of mGluRs have been shown to play an important role in the induction of plasticity at a variety of synapses in the CNS (Bortolotto et al 1999; Linden 1999; Nevian & Sakmann 2006).

Recent studies have suggested the role of $[Ca^{2+}]_i$ in the plasticity of ionotropic glutamate receptor (iGluR)-mediated transmission onto DA neurons (Jones & Bonci 2005). In particular, LTP can be induced in a manner dependent on bursts of APs (Liu et al 2005), whereas activation of mGluRs has been implicated in the induction of LTD (Bellone & Luscher 2005), although other forms of synaptic plasticity have been reported using different induction protocols (Gutlerner et al 2002; Ungless et al 2001). In light of the opposing roles played by AP- and mGluR-induced Ca^{2+} signals, it is likely that these two types of signals can be differentially regulated.

1.2.3.2 Regulation of Ca^{2+} signaling by sustained activation of PI-coupled receptors

As mentioned previously, transient activation of PI-coupled receptors produces phasic rise in intracellular Ca^{2+} mobilizing messengers, which generates large propagating Ca^{2+} wave in DA neurons. Meanwhile, DA neurons may also be under the influence of tonic activation of these receptors caused by ambient low level of neurotransmitters (Descarries 1998; Zoli et al 1998). It has been shown that tonic activation of mGluRs, AChRs, and α1Rs suppresses messenger-induced Ca^{2+} release from intracellular stores DA neurons, thereby inhibiting mGluR-mediated IPSP (Fiorillo & Williams 1998; 2000; Paladini & Williams 2004). Thus non-selective adrenergic receptor agonist NE enhances burst firing of DA neurons in vivo (Shi et al 2000). Ca^{2+} store depletion has been proposed as one of the mechanisms mediating this effect (Paladini et al 2001).

On the other hand, AP-evoked Ca^{2} responses have been shown being enhanced by tonic activation of mGluRs and mAChRs in hippocampal and cortical pyramidal neurons (Nakamura et al 1999; Power & Sah 2002; Yamada et al 2004), and by α1AR activation in dorsal raphe neurons (Pan et al 1994). How AP-induced Ca^{2+} signals are regulated by PI-coupled receptor activation in DA neurons is unknown.

1.2.4 PI-COUPLED RECEPTOR ACTIVATION AND DRUG ADDICTION

At behavioral level, substantial evidence suggests that activation of PI-coupled receptors plays an important role in brain reward function and drug addiction. DA neurons receive multiple inputs of neurotransmitters activating PI-coupled receptors, including glutamate, acetylcholine, norepinephrine, 5-HT, and some types of neuropeptides (Borgland et al 2006; Fiorillo & Williams 1998; 2000; Paladini & Williams 2004; Walsh & Cunningham 1997).

The essential role of mGluR activation in addiction has been best demonstrated in studies showing that intra-VTA application of mGluR antagonist MCPG blocked amphetamine-induced behavioral sensitization (Kim & Vezina 1998), whereas intra-VTA administration of mGluR agonist t-ACPD induced behavioral sensitization to subsequent systemic cocaine injection (Dunn et al 2005). These effects are likely mediated by mGluR-mediated Ca^{2+} signaling in DA neurons, because microinjection of CaM-KII inhibitor KN-93 into VTA blocked t-ACPD-induced behavioral sensitization to systemic cocaine (Dunn et al 2005). Furthermore, systemic administration of selective mGluR1 antagonist CPCCOET dose-dependently inhibits ethanol self-administration and ethanol-induced CPP (Lominac et al 2006).

Amphetamine has been shown to activate α1ARs by releasing DA and NE in the midbrain, which facilitates burst firing of DA neurons by causing membrane depolarization and by suppressing mGluR-mediated IPSP (Paladini et al 2001; Shi et al 2000). Systemic or prefrontal local injection of a1AR antagonist

prazosin suppresses amphetamine- and cocaine-induced locomotor stimulation (Darracq et al 1998; Dickinson et al 1988; Wellman et al 2002). α1AR deficient mice are severely affected in their locomotor response and behavior sensitization to both psychostimulants and opiates (Drouin et al 2002) . Furthermore, prazosin blocks enhanced cocaine self-administration induced by cocaine pre-exposure (Zhang & Kosten 2006).

DA neurons receive cholinergic input from PPTg (Futami et al 1995). Lesion of PPTg with ibotenate disrupts the learning of associations between conditioned stimuli and primary rewards (Inglis et al 1994). Intra-VTA administration of acetylcholine enhances lever pressing for brain stimulation reward (Redgrave & Horrell 1976), while mAChR antagonist atropine inhibits it (Yeomans & Baptista 1997). Intra-VTA blockade of mAChRs with scopolamine also inhibits food-related reward learning (Sharf & Ranaldi 2006). Furthermore, rats self-administer non-selective acetylcholine receptor agonist carbachol into VTA in an mAChR-dependent fashion (Ikemoto & Wise 2002). And finally, genetically knocking out M5 mAChR, the only type of mAChR expressed in DA neurons (Vilaro et al 1990; Weiner et al 1990), inhibits self-administration of cocaine and disrupts cocaine- and morphine-induced CPP in mice (Basile et al 2002; Fink-Jensen et al 2003).

Taken together, the critical role of PI-coupled receptor activation in reward and addictive behaviors has been well established. However, the linkage

between the action of PI-coupled receptors in regulating Ca^{2+} signals at the cellular level and their behavioral effects in drug addiction is still missing.

1.3 Hypothesis and specific aims

As stated above, strengthened synaptic connection between glutamatergic input and DA neurons has been proposed to be a candidate cellular mechanism that mediates drug-induced behavioral sensitization, an animal model for long-lasting drug craving in human addicts (Jones & Bonci 2005; Kauer 2004). Drugs of abuse have been shown to shift the balance of plasticity toward potentiation in these synapses either by inhibiting LTD or by facilitating LTP (Jones et al 2000; Liu et al 2005; Saal et al 2003). Since the bi-directionality of synaptic plasticity is controlled by the amplitude and spatiotemporal profile of $[Ca^{2+}]_i$ (Artola & Singer 1993; Linden 1994; Linden & Connor 1995; Lisman 1989), it is plausible that addictive drugs may regulate DA neuron synaptic plasticity by inhibiting LTD-associated Ca^{2+} signals while enhancing LTP-associated Ca^{2+} signals. Postsynaptic AP- and mGluR-induced Ca^{2+} signals have been implicated in the induction of LTP (Liu et al 2005) and LTD (Bellone & Luscher 2005), respectively in DA neurons. Thus an intriguing hypothesis would be that addictive drugs could differentially regulate these two 'opposing' Ca^{2+} signals. If true, this unique mechanism will add significantly to our understanding of neuronal mechanisms underlying drug addiction at cellular and molecular levels.

One pathway by which addictive drugs can affect intracellular Ca^{2+} signaling is to stimulate PI-coupled neurotransmitter receptors, such as mGluRs,

α1ARs, and mAChRs. Indeed, activation of these receptors is essential for psychostimulant-induced behavioral sensitization (Darracq et al 1998; Dickinson et al 1988; Kim & Vezina 1998; Wellman et al 2002) and reinforcement (Basile et al 2002; Fink-Jensen et al 2003; Zhang & Kosten 2006). Previous studies have shown that tonic activation of PI-coupled receptors suppresses phasic mGluR-induced Ca^{2+} signals in DA neurons (Fiorillo & Williams 1998; 2000; Paladini & Williams 2004). However, how AP-induced Ca^{2+} signals are regulated in DA neurons by activation of these receptors remains unknown. The overall goal of this dissertation is to examine the effects of sustained activation of PI-coupled neurotransmitter receptors on AP-evoked Ca^{2+} responses in DA neurons, and to elucidate the cellular mechanisms underlying these effects.

The first specific aim is to examine the effects of tonic PI-coupled receptor activation on AP- and mGluR-induced Ca^{2+} responses in the same neuron. Ca^{2+} responses will be assessed by Ca^{2+}-sensitive SK channel-mediated outward currents in voltage clamp and by fluorescence change of Ca^{2+} indicator dyes (Morikawa et al 2003; Paladini et al 2001). APs and phasic activation of mGluRs will be evoked by injecting depolarizing pulses and by iontophoretic application of aspartate (Morikawa et al 2003), respectively. Tonic activation of PI-coupled receptors will be induced by bath application of selective agonists or by prolonged low-intensity stimulation of afferent fibers.

The second specific aim is to characterize intracellular mechanisms mediating the effects observed in aim 1, and further examine the regulation of

AP-induced Ca^{2+} signals by physiological firing activity of DA neurons. We will test the involvement of Ca^{2+} stores by depleting stores with bath application cyclopiazonic acid (CPA) (Seidler et al 1989) and ryanodine (Smith et al 1988; Zucchi & Ronca-Testoni 1997). The IP_3R antagonist heparin (Ghosh et al 1988) and RyR antagonist ruthenium red (Ma 1993; Smith et al 1985; Smith et al 1988) will be applied intracellularly to examine the involvement of these two Ca^{2+} mobilizing pathways. Flash photolysis of caged compounds will be used to deliver chemicals into the cell and to evoke Ca^{2+} release from intracellular stores (Morikawa et al 2000). To examine the firing activity-dependent regulation of AP-induced Ca^{2+} signals, APs will be evoked in physiologically relevant 2 Hz trains (Chiodo 1988; Grace & Bunney 1984b). Its effect on single AP- and burst-induced Ca^{2+} signals will be studied.

The third specific aim is to examine the effect of amphetamine on AP burst-induced Ca^{2+} signals. Amphetamine is known to release DA, activating both D2 autoreceptors (Mercuri et al 1989) and α1ARs (Paladini et al 2001; Shi et al 2000) in DA neurons. D2R activation inhibits basal firing of DA neurons, but its effect on burst firing is elusive. We will first test the effect of amphetamine on basal and burst firing with cell-attached firing recordings. Burst will be induced by iontophoretic application of aspartate (Morikawa et al 2003). We will then examine the effect of amphetamine-induced firing pattern changes on burst-induced Ca^{2+} responses. More specifically, we will evoke APs in patterns (basal + burst) that mimic the control firing and the firing under the impact of

amphetamine, and compare the size of burst-induced I_{AHP}. Finally, selective α1AR agonist phenylephrine will be bath applied to examine the amphetamine-induced combinational effect of D2R and α1AR activation on burst-induced Ca^{2+} responses.

CHAPTER 2: MATERIALS AND METHODS

2.1 Slices and solutions

Horizontal slices (200-220 μm) of the ventral midbrain were prepared from male Sprague Dawley rats (3-6 weeks old, except for those experiments studying spontaneous miniature outward currents [SMOCs], where neonatal rats [6-14 days old] were used), as described previously (Morikawa et al 2003). Slices were maintained at 35°C and perfused at a rate of 2-3 ml/min with physiological saline containing (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, 21.4 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2 (pH 7.4, 300 mOsm/kg). Unless noted otherwise, pipette solutions used for whole-cell and cell-attached recordings contained (in mM): 115 K-methylsulfate, 20 KCl, 1.5 $MgCl_2$, 10 HEPES, 0.025 EGTA, 2 Mg-ATP, 0.2 Na_2-GTP, and 10 Na_2-phosphocreatine (pH 7.3, 280 mOsm/kg).

2.2 Electrophysiological recordings

All recordings were performed in DA neurons, which were identified by their large cell bodies (>20 μm) visualized with IR/DIC optics, spontaneous firing at 1-5 Hz, and the presence of large hyperpolarization-activated I_h currents (>200

pA). Most (~90%) of the recordings were made in the SNc, while the remainder was in the VTA. Whole-cell pipettes had resistances of 1.5-2.5 MΩ. Voltage-clamp recordings were made at a holding potential of -62 mV, corrected for a liquid junction potential of 7 mV, unless stated otherwise. Series resistance (~10-20 MΩ) was continuously monitored but left uncompensated. MultiClamp 700A or 700B amplifiers (Axon Instruments, Foster City, CA) were used to record the data, which were filtered at 1-2 kHz, digitized at 2-5 kHz, and collected using AxoGraph 4.9 (Molecular Devices) or AxoGraph X (AxoGraph Scientific).

Iontophoretic pipettes (~100 MΩ) containing L-aspartate (1 M, pH 7.4) were placed within 5 µm of the soma or proximal dendrites. Iontophoretic pulses (50-200 nA, 30-200 ms, 0-5 nA backing current) were applied once per minute. Synaptic responses were evoked with a bipolar tungsten electrode (tip separation 100 µm) placed at 50-100 µm rostral to the recorded cell. In order to isolate mGluR-mediated responses, these experiments were done in slices treated with 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 µM), (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801; 50 µM), picrotoxin (100 µM), CGP 55845 (10 µM), and eticlopride (100 nM) to block AMPA, NMDA, $GABA_A$, $GABA_B$, and DA D2 receptors, respectively.

In whole-cell current clamp recordings, hyperpolarizing currents were injected to maintain the membrane potential at ~-60 mV. APs were evoked by 2-ms depolarizing current injection.

Spontaneous AP firing was monitored using either perforated-patch or cell-attached recording configurations. Perforated-patch pipettes were tip-filled with gramicidin (50-250 μg/ml) in a solution containing (in mM): 135 KCl, 10 HEPES. The AHP amplitude was measured by calculating the difference between the AP threshold, defined as the voltage where dV/dt exceeded 10 V/s, and the hyperpolarization peak of the AHP. The firing frequency within aspartate-induced bursts was obtained by calculating the average of the second and third interspike intervals after aspartate iontophoresis. For cell-attached recordings, traces of dV/dt are shown for clarity.

2.3 Ca^{2+} imaging

Fluorescent imaging of $[Ca^{2+}]_i$ was made using fluo-5F or fluo-4FF as Ca^{2+} indicators. These indicator dyes were loaded into the cell via the whole-cell pipette. At least 20 min were waited before pictures were taken to allow the maximal diffusion of indicator dyes. Images were taken at 15-20 Hz using the Olympus Disk Spinning Unit confocal imaging system. Ca^{2+} signals from selected regions of interests (ROIs) were expressed as $\%\Delta F/F = 100 \times (F - F_{baseline})/(F_{baseline} - F_{background})$.

2.4 Flash photolysis

A 1-ms UV pulse was applied to elicit photolysis of caged IP_3 or caged Ca^{2+} and the resulting SK-mediated outward current was measured. Our previous study demonstrated roughly linear relationship between the IP_3-evoked current and $[Ca^{2+}]_i$ over a wide range in DA neurons (Morikawa et al 2000). The peak of the IP_3-evoked current trace was not rounded even with a supra-maximal intensity of UV pulse, suggesting that SK channels were not saturated by Ca^{2+}. The voltage of the capacitor supplying current to the flash lamp was held constant at 300 V and the capacitance of the capacitor was varied (50-4000 μF) to control the intensity of UV pulse, which is known to be proportional to the concentration of compounds photolytically released (Khodakhah & Ogden 1993). Caged IP_3 (100-200 μM) was loaded into the cell through the whole-cell pipette. For caged Ca^{2+} experiments, DM-nitrophen (1.5 mM) and $CaCl_2$ (1.2 mM) were added to the whole-cell pipette.

2.5 Drugs

DNQX, MK-801, cyclopiazonic acid (CPA), CGP 55845, LY 367385, and (S)-3,5-dihydroxyphenylglycine (DHPG) were obtained from Tocris Cookson (Ellisville, MO). Heparin, ruthenium red, ryanodine, and DM-nitrophen were purchased from Calbiochem (La Jolla, CA). Tetrodotoxin (TTX) was obtained from Alomone Labs (Jerusalem, Israel). Fluo-5F, fluo-4FF, and caged IP_3 were

purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma/RBI (St. Louis, MO).

2.6 Data analysis

Data are expressed as means ± SEM. Statistical significance was determined by Student's t test or ANOVA followed by Bonferroni's post-test. The difference was considered significant at $p < 0.05$.

CHAPTER 3: RESULTS

3.1 Aim1: Differential effects of PI-coupled receptor activation on AP- and mGluR-induced Ca^{2+} signals

3.1.1 APs TRIGGER IP_3R- AND RyR-MEDIATED CICR IN DA NEURONS

AP-induced Ca^{2+} transients can be amplified by CICR from intracellular stores via IP_3Rs and/or RyRs in a variety of neurons (Berridge 1998). Ca^{2+} release from intracellular stores has been suggested to contribute to the generation of AHPs in DA neurons (Wolfart & Roeper 2002). However, it is not clear which receptors, IP_3Rs or RyRs, are mediating AP-evoked CICR in DA neurons.

3.1.1.1 Evoking AP-dependent, Ca^{2+} sensitive K^+ currents with voltage-clamp recordings

Whole-cell voltage clamp recordings were made from DA neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). In order to assess AP-induced Ca^{2+} signals, a 2-ms depolarizing test pulse of 30-60 mV was applied from a holding potential of -62 mV to evoke an unclamped AP. This produced an outward tail current lasting 150-300 ms, which was inhibited by apamin (50-100 nM), an SK channel blocker (n = 33; Fig. 3.1A). The apamin-

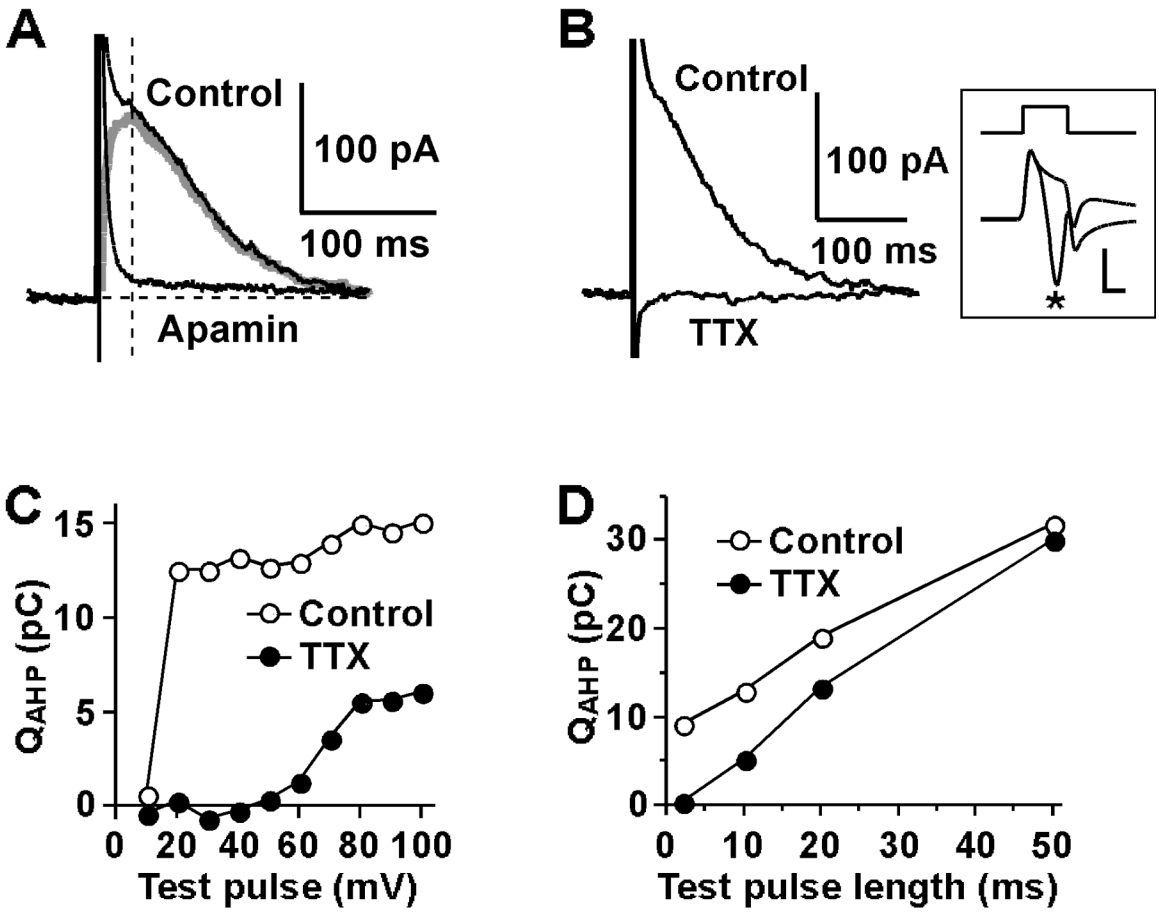

Figure 3.1. Evoking AP-induced, SK channel-mediated I_{AHP}.

(A) Representative traces of outward tail currents caused by an unclamped AP, evoked by a 2-ms depolarizing pulse, in control and in apamin (100 nM). Apamin selectively eliminated the slow component of the tail current. The gray trace represents the apamin-sensitive current (I_{AHP}) obtained by current subtraction. The vertical dashed line is drawn at 20 ms after the test pulse. (B) TTX (1 μM) abolished both the fast and slow components of the outward tail current. Currents elicited during the 2-ms test pulse are shown in the inset. Asterisk depicts the unclamped action current in control, which was blocked by TTX. Scale bars in the inset correspond to 1 ms and 1 nA. (C) QAHP is plotted versus the amplitude of the test pulse in a cell shown in (B). TTX largely eliminated QAHP at test pulse amplitudes up to 60 mV. (D) QAHP is plotted versus the duration of the test pulse in a different cell. The test pulse amplitude was set at 50 mV. Increasing the test pulse duration to 10 ms or above added a TTX-insensitive component.

sensitive component, termed I_{AHP}, peaked at ~20 ms after the test pulse, while the transient outward current insensitive to apamin mostly decayed within 20 ms. Accordingly, we calculated the integral of the outward current from 20 ms to 300-600 ms after the test pulse to assess the charge transfer representing I_{AHP} (called Q_{AHP} hereafter). I_{AHP} was evoked in an all-or-none fashion when the amplitude of the test pulse was varied up to 50-60 mV, and was abolished by TTX (1 μM, n =11; Fig. 3.1B-D). Further increasing the test pulse amplitude (n = 4) or prolonging the test pulse duration (n = 4) added a TTX-insensitive component to I_{AHP}. We also monitored the $[Ca^{2+}]_i$ rise evoked with 2-ms test pulses at different amplitudes by measuring fluorescence change in fluo-5F (50 μM) filled neurons. In these experiments, depolarizing pulses were applied in a train of 5 pulses at 20 Hz to mimic burst firing of DA neurons. $[Ca^{2+}]_i$ rise induced by 30 or 50 mV depolarizing pulses was completely eliminated by TTX (1 μM), while 100 mV test pulse produced a significant TTX-insensitive component (n = 2; Fig. 3.2). Thus, we routinely used a 2-ms depolarizing pulse of 30-60 mV to evoke SK-mediated I_{AHP} which is triggered solely by unclamped AP-dependent Ca^{2+} influx.

3.1.1.2 AP-evoked Ca^{2+} influx is amplified by CICR through both IP_3Rs and RyRs

Consistent with the previous report (Wolfart & Roeper 2002), bath application of cyclopiazonic acid (CPA; 10-20 μM), which depletes endoplasmic reticulum Ca^{2+} stores (Smith et al 1988), depressed Q_{AHP} by 43 ± 3% (n = 7; Fig.

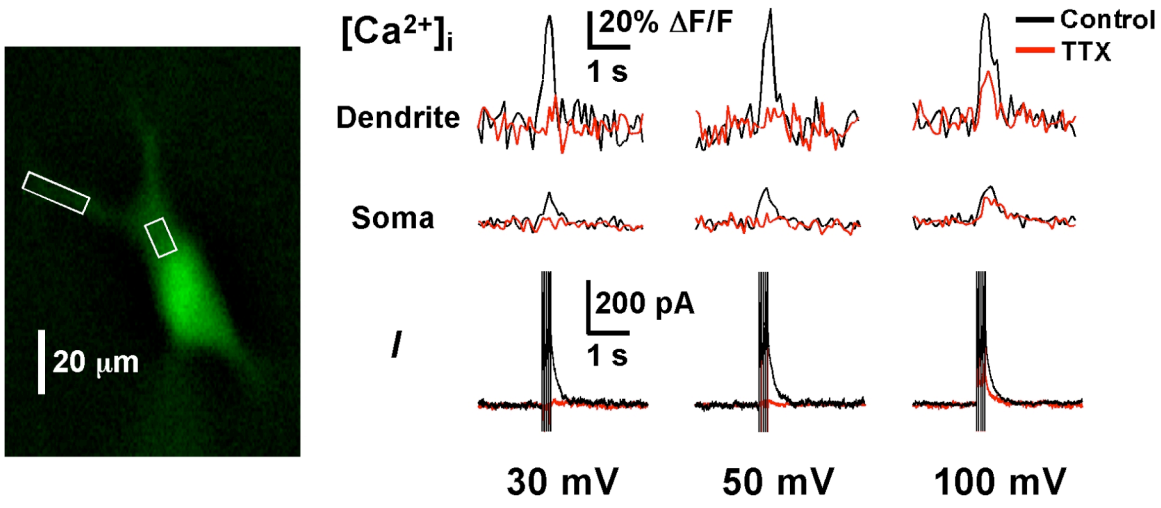

Figure 3.2. Effect of TTX on burst-induced Ca^{2+} transients.

A confocal fluorescent image of a DA neuron loaded with fluo-5F (50 mM) is shown on the left. Trains of 5 depolarizing test pulses (2-ms duration) of different amplitudes (30, 50, and 100 mV) were applied at a frequency of 20 Hz to evoke bursts of unclamped APs. Fluorescence changes were measured at the ROIs indicated on the left panel in control and in TTX (1 mM), while currents were recorded in the same cell (bottom traces). Note that TTX blocked burst-induced fluorescence changes at the soma and the proximal dendrite, as well as $I_{AHP\text{-}burst}$, when the test potential amplitude was 30 or 50 mV, whereas significant TTX-insensitive components were present with a test pulse of 100 mV.

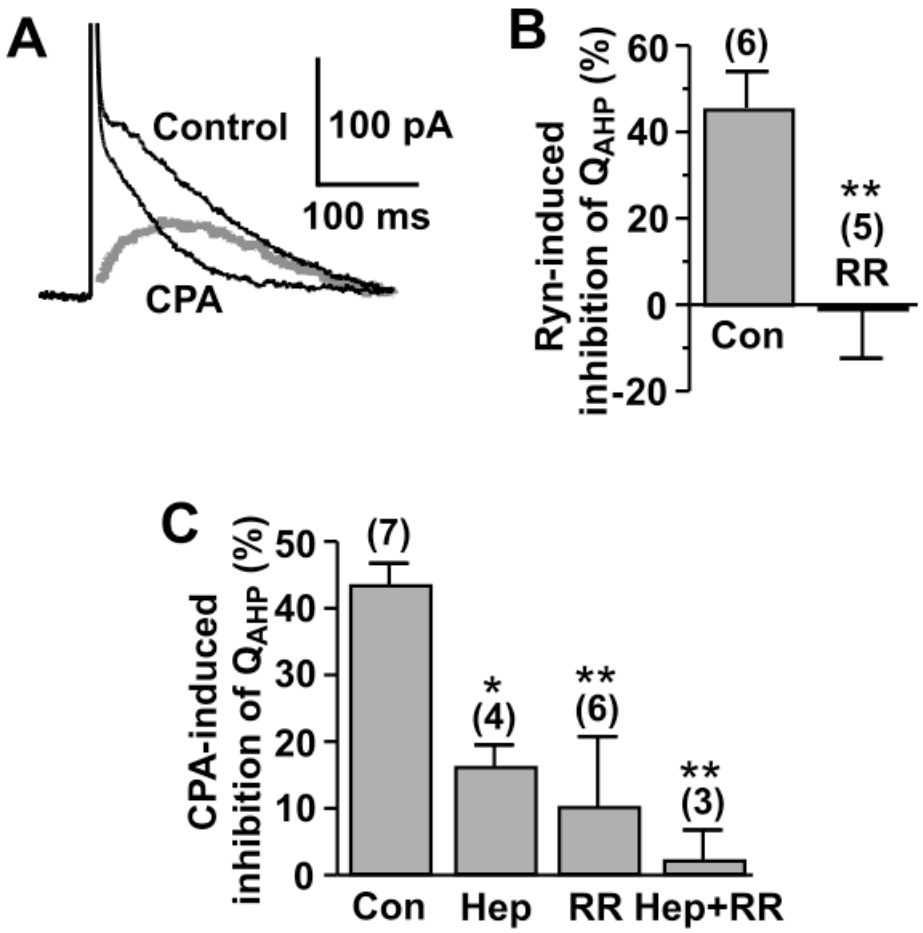

Figure 3.3. APs trigger CICR via both IP_3Rs and RyRs.

(A) Representative traces illustrating the effect of CPA (20 μM) on I_{AHP}. The gray trace depicts the CPA-sensitive component of I_{AHP}. (B) Summary bar graph depicting the effect to ryanodine (20 μM) with a control internal solution or ruthenium red (200 μM). (C) Summary bar graph plotting the effect of CPA in cells recorded with a control internal solution, heparin (Hep; 1 mg/ml), ruthenium red (RR; 200 μM), and both of these drugs. *$p < 0.05$, **$p < 0.01$ vs. control.

3.3A,C). The CPA-sensitive component of I_{AHP} peaked at 50-100 ms after the test pulse. Ryanodine (20 μM), which locks RyR channels in a sub-conductance open state and thus depletes Ca^{2+} stores expressing RyRs (Zucchi & Ronca-Testoni 1997), also decreased Q_{AHP} by 45 ± 9% (n = 6, Fig. 3.3B). Intracellular application of heparin (1 mg/ml), an IP_3R antagonist (Ghosh et al 1988), or ruthenium red (200 μM), a RyR antagonist (Zucchi & Ronca-Testoni 1997), significantly reduced the effect of CPA (Fig. 3.3C). The effect of ryanodine was completely eliminated by ruthenium red (n = 5, Fig. 3.3B). These results demonstrate that both IP_3Rs and RyRs are involved in CICR triggered by AP-induced Ca^{2+} influx. In line with the contribution of intracellular stores to AP-induced Ca^{2+} signals, CPA reduced the AHP amplitude of spontaneously firing DA neurons from 30 ± 3 mV to 26 ± 2 mV (n = 3, perforated-patch recordings, $p < 0.05$; Fig. 3.4A) and increased the firing rate from 2.2 ± 0.2 Hz to 2.7 ± 0.3 Hz (n = 9, cell-attached or perforated-patch recordings, $p < 0.05$; Fig. 3.4B).

3.1.2 Differential regulation of AP- and phasic mGluR-induced Ca^{2+} signals

Tonic activation of PI-coupled neurotransmitter receptors, such as mGluRs, α1ARs, and mAChRs, inhibits intracellular Ca^{2+} release produced by strong, phasic activation of these receptors, likely via interfering Ca^{2+} release from internal stores (Fiorillo & Williams 2000; Morikawa et al 2003; Paladini et al

2001). We thus tested whether sustained activation of these receptors could also affect the Ca^{2+} store-dependent component of I_{AHP}.

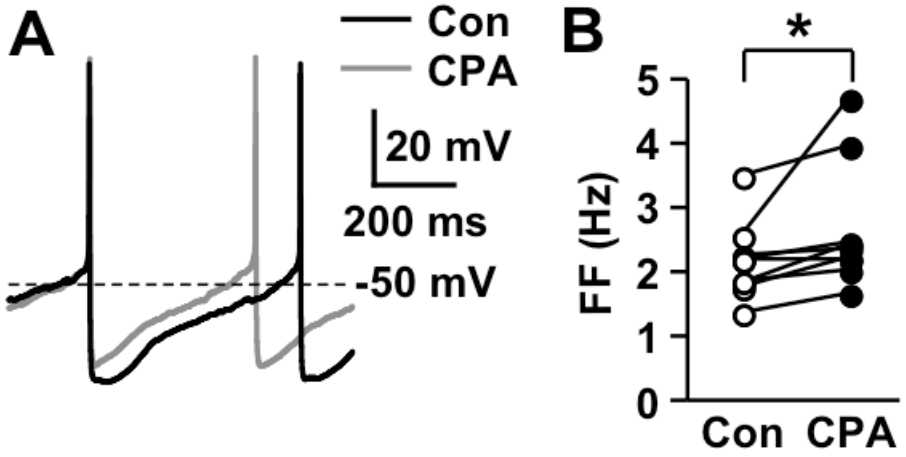

Figure 3.4. CPA reduces AHP amplitude and increases spontaneous firing frequency.

(A) Representative traces of firing in control and in CPA (20 mM) recorded with a perforated-patch configuration. CPA reduced the AHP amplitude and the interspike interval. (B) Firing frequency (FF) of individual cells in control and in CPA. *$p < 0.05$.

3.1.2.1 Agonist-induced tonic activation of PI-coupled receptors augments AP-induced Ca^{2+} transients while suppressing mGluR-induced Ca^{2+} signals

Bath perfusion of mGluR agonist DHPG (1 μM, n = 4), α1AR agonist phenylephrine (10 μM, n = 7), and mAChR agonist muscarine (1 μM, n = 7) all produced reversible inhibition of the mGluR-mediated outward current (I_{mGluR}) evoked by aspartate iontophoresis in the presence of iGluR antagonists (Fig. 3.5A,C). In contrast, DHPG dramatically augmented I_{AHP} by adding a slow component, which lasted up to 600 ms (Fig. 3.5A). This resulted in 108 ± 17% increase in Q_{AHP} (n = 21; Fig. 3.5C). Furthermore, superfusion of phenylephrine and muscarine also increased Q_{AHP} by 65 ± 10% (n = 11) and 116 ± 25% (n = 4), respectively (Fig. 3.5B,C). DHPG (n = 2) and phenylephrine (n = 2) also increased the AHP amplitude measured in whole-cell current clamp by ~30% (Fig. 3.5B).

We next monitored $[Ca^{2+}]_i$ by the fluorescence of fluo-5F (50 μM; K_d = 2.3 μM) loaded into the cell. In these experiments, we evoked a train of 5 unclamped APs at 20 Hz to mimic burst firing of DA neurons. Aspartate iontophoresis was applied at least 10 s after the burst to monitor both burst- and mGluR-induced Ca^{2+} responses in the same cell (Fig. 3.6A). The burst of APs produced a rise in $[Ca^{2+}]_i$ and a summating outward current with a prolonged tail. The total charge transfer of this outward current, termed $Q_{AHP\text{-}burst}$, was calculated after removing a

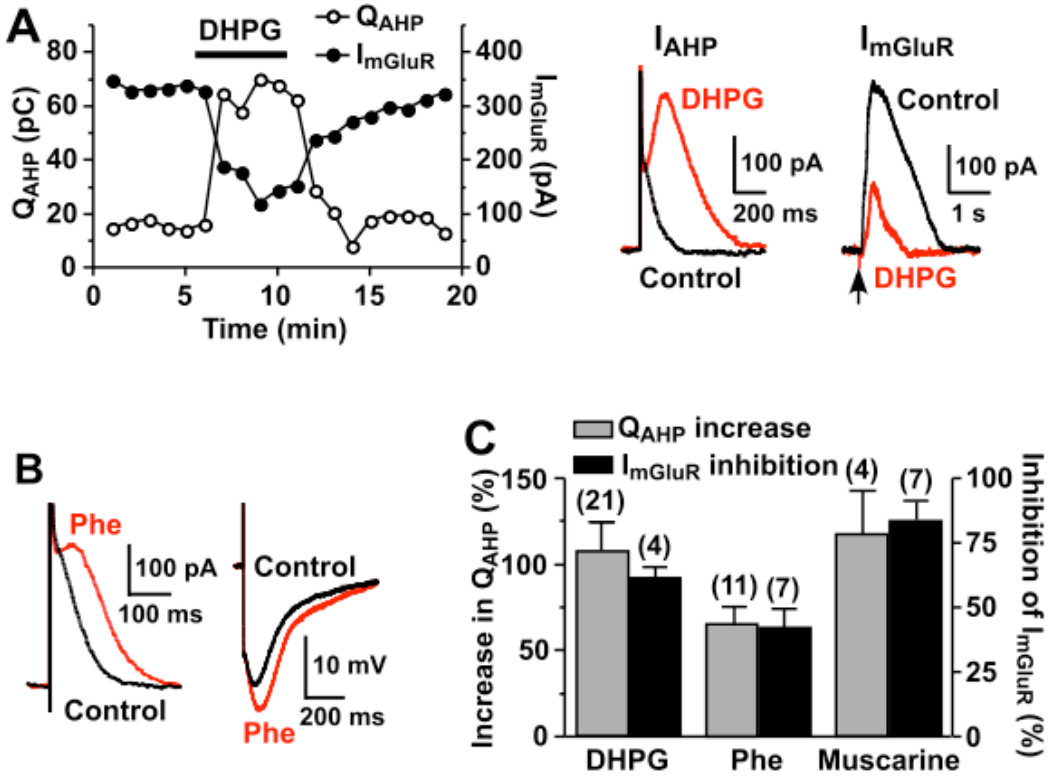

Figure 3.5. Sustained activation of PI-coupled receptors induced by bath application of selective agonists differentially regulates I_{AHP} and I_{mGluR}.

(A) Time graph depicting the differential effects of DHPG (1 μM) on Q_{AHP} and I_{mGluR} recorded in the same cell. Representative traces of I_{AHP} and I_{mGluR} in control and in DHPG from the same experiment are shown on the right. I_{mGluR} was evoked by iontophoretic application of aspartate (100 ms) at the time indicated by the arrow. (B) Left: Representative traces of I_{AHP} in control and in phenylephrine (Phe; 10 μM). Right: Representative traces illustrating that phenylephrine also augmented AHPs recorded in whole-cell current clamp. AP was evoked by a 2-ms current injection. (C) Summary bar graph showing that DHPG, phenylephrine, and muscarine all increased I_{AHP} and inhibited I_{mGluR}.

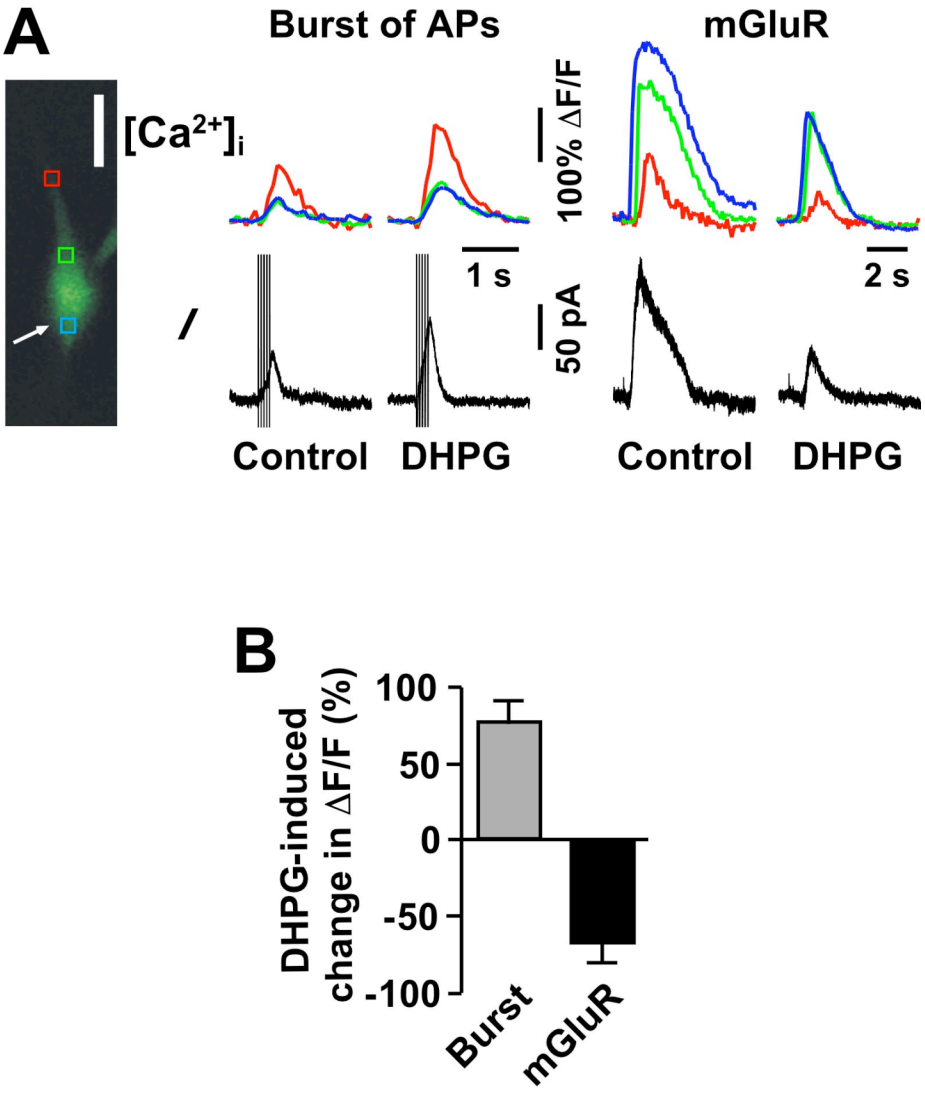

Figure 3.6. DHPG differentially regulates Burst- and mGluR-induced Ca^{2+} signals.

(A) DHPG (1 μM) enhanced the Ca^{2+} signal induced by a train of APs while suppressing the phasic mGluR-mediated Ca^{2+} wave. A confocal fluorescence image of a DA neuron loaded with fluo-5F (50 μM) is shown on the left. The scale bar is 20 μm. A train of 5 test pulses at 20 Hz was used to evoke a burst of 5 unclamped APs. Aspartate was iontophoresed at the soma, as indicated by the arrow. Fluorescence changes were measured at the ROIs indicated by the boxes with different color codes. $I_{AHP\text{-}burst}$ and I_{mGluR} evoked concomitantly are also shown. (B) Summary bar graph depicting the effects of DHPG on burst- and mGluR-induced Ca^{2+} signals measured in dendrites ~20-50 μm away from the soma.

20-ms window following each test pulse to isolate the apamin-sensitive component (see Fig. 3.1A). The burst-induced Ca^{2+} signal appeared simultaneous throughout the cell but was invariably larger in proximal dendrites (~20-50 μm from the soma) than in the soma. TTX (1 μM) completely eliminated burst-induced fluorescence change in dendrites and also reduced that in the soma by ~80% (n =5), consistent with the major contribution of unclamped APs. Aspartate iontophoresis induced an mGluR-mediated wave of Ca^{2+} starting at the site of application and propagating in a diffusive fashion, i.e., the magnitude and rate of rise in $[Ca^{2+}]_i$, as well as the speed of propagation, declined with wave propagation, as reported previously (Morikawa et al 2003; Paladini & Williams 2004). Bath application of DHPG (1 μM) increased burst-induced fluorescence change by 75 ± 15% and decreased mGluR-induced fluorescence change by 66 ± 12% when measured at proximal dendrites (n = 5; Fig. 3.6B).

3.1.2.2 Weak synaptic stimulation-induced tonic activation of mGluRs augments burst-induced Ca^{2+} signals.

We further induced sustained activation of mGluRs by weak synaptic stimulation of glutamatergic fibers (60 stimuli at 50 Hz, starting 1 s before the onset of the burst), which evoked no detectable Ca^{2+} transient or outward current by itself (Fig. 3.7). Synaptic stimulation increased burst-induced fluorescence change at the proximal dendrite close to the stimulating electrode by 59 ± 6% (n = 2) and $Q_{AHP\text{-}burst}$ by 60 ± 11% (n =3). The facilitatory effect of synaptic

stimulation was blocked by bath application of LY367385 (50 μM), an $mGluR_1$ antagonist.

Altogether, these results show that tonic activation of PI-coupled receptors augments AP-induced Ca^{2+} transients and suppresses phasic mGluR-mediated Ca^{2+} responses in DA neurons.

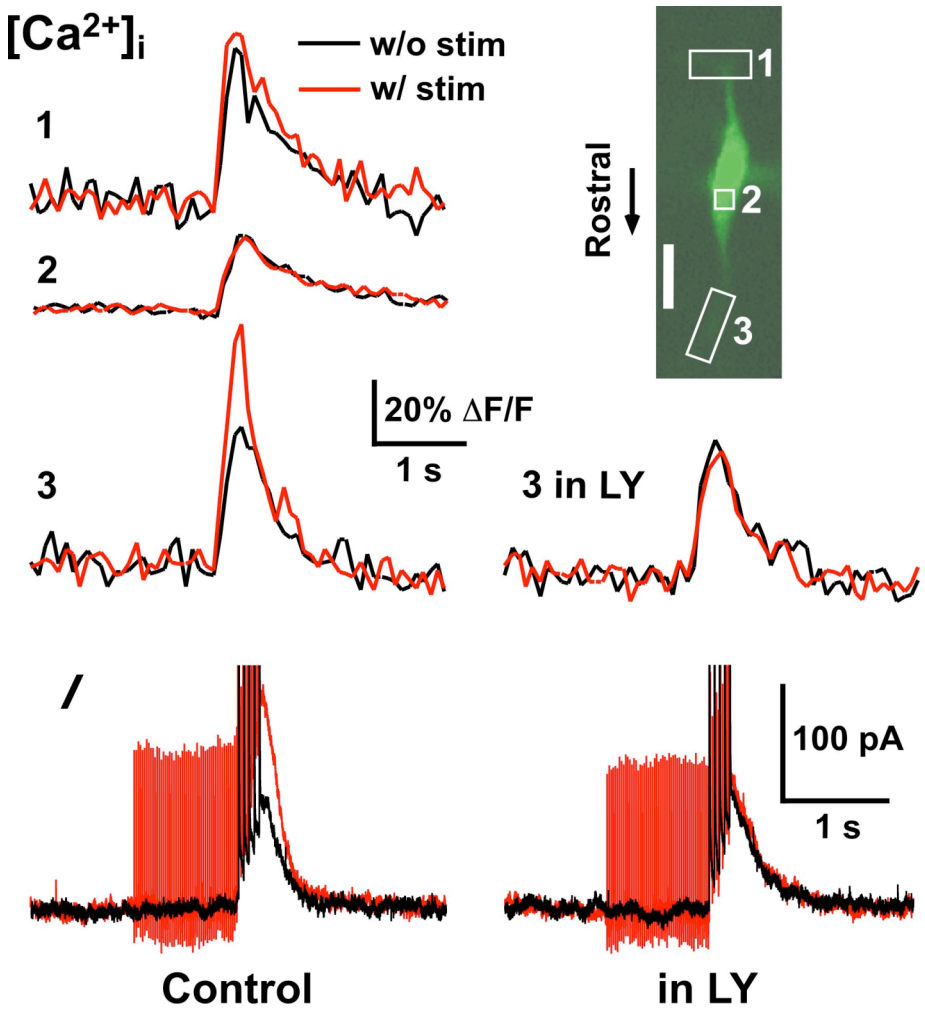

Figure 3.7. Weak synaptic stimulation-induced activation of mGluRs facilitates burst-induced Ca^{2+} transients.

A confocal fluorescence image of a DA neuron loaded with fluo-5F (50 μM) is shown on the right. A bipolar stimulating electrode was placed ~50 μm rostral to the cell. A train of 5 depolarizing pulses at 20 Hz, evoking a burst of APs, was applied alone (black) or paired with synaptic stimulation (red). The synaptic stimulation consisted of 60 stimuli at 50 Hz starting 1 s before the onset of the burst. The stimulation intensity was adjusted so that synaptic stimulation alone did not induce detectable fluorescence changes. Fluorescence changes were measured at the ROIs indicated by the numbered boxes. $I_{AHP\text{-}burst}$ evoked concomitantly is also shown at the bottom. Synaptic stimulation enhanced $I_{AHP\text{-}bust}$ and the burst-induced Ca^{2+} signal at the rostral dendrite close to the stimulating electrode. This enhancement was blocked by LY367385 (50 μM).

3.2 Aim2: Intracellular mechanisms mediating the differential regulation of AP- and phasic mGluR-induced Ca^{2+} signals

3.2.1 IP_3Rs, but not RyRs, mediate PI-coupled receptor-induced enhancement of I_{AHP} and inhibition of I_{mGluR}

G protein-coupled metabotropic receptors can produce two Ca^{2+}-mobilizing messengers, IP_3 and cADPR, which act on IP_3Rs and RyRs, respectively (Cancela 2001). To test the involvement of these two messenger pathways, we used heparin (1 mg/ml) to block the IP_3-IP_3R pathway and ruthenium red (200 μM) to block the cADPR-RyR pathway. DHPG (1 μM)-induced enhancement of I_{AHP} and suppression of I_{mGluR} were both dramatically attenuated by heparin but not by ruthenium red (Fig. 3.8). Furthermore, the effects of phenylephrine (10 μM) and muscarine (1 μM) on I_{AHP} and I_{mGluR} were also nearly abolished by heparin (n = 3 for each, data not shown). Altogether, these data suggest that an increase in IP_3 tone mediates both PI-receptor activation-induced facilitation of I_{AHP} and suppression I_{mGluR}.

3.2.2 PI-coupled receptor activation inhibits I_{mGluR} via depleting Ca^{2+} stores

Although it has been shown that tonic PI-coupled receptor activation inhibits mGluR-induced Ca^{2+} release from intracellular stores in DA neurons

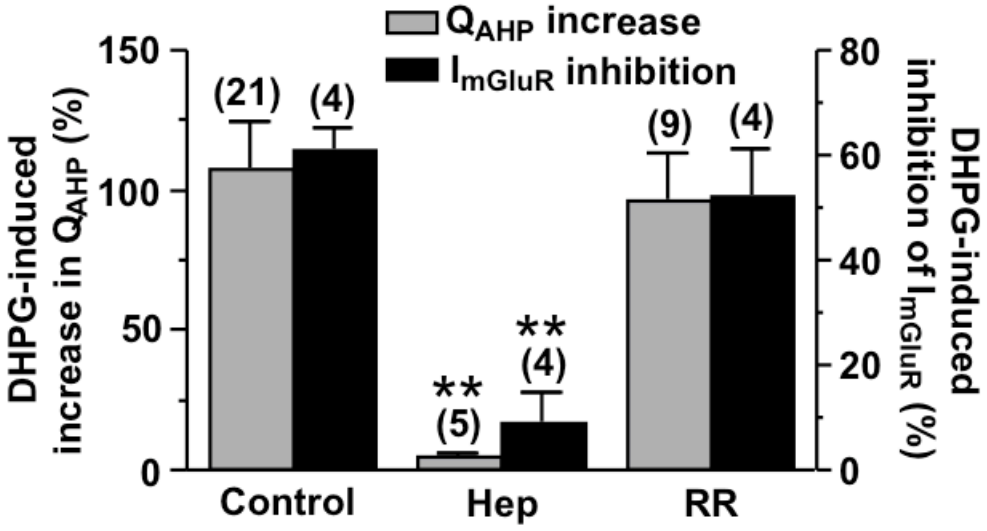

Figure 3.8. IP_3Rs, but not RyRs, mediate differential regulation of AP- and phasic mGluR-induced Ca^{2+} signals.

Summary bar graph showing the effects of DHPG (1 μM) on Q_{AHP} and I_{mGluR} in cells recorded with a control internal solution, heparin (Hep; 1 mg/ml), ruthenium red (RR; 200 μM). **p < 0.01 vs. control.

(Fiorillo & Williams 1998; Paladini & Williams 2004), the intracellular mechanism remains unclear. Two potential mechanisms have been proposed: IP3R desensitization and depletion of Ca^{2+} stores (Paladini et al 2001). One way to discriminate between these two mechanisms is to examine the effects of PI-coupled receptor activation on IP_3R- and RyR-mediated Ca^{2+} release separately, because store depletion should cause inhibition in both IP_3R- and RyR-mediated Ca^{2+} release, whereas IP_3R desensitization would only affect IP_3R-mediated Ca^{2+} release.

3.2.2.1 PI-coupled receptor activation inhibits both IP_3R- and RyR-mediated Ca^{2+} release

It has been shown that PI-coupled receptor agonists suppresses Ca^{2+} release mediated by IP_3Rs in DA neurons (Paladini et al 2001). However, the effect of PI-coupled receptor activation on RyR-mediated Ca^{2+} release remains unknown. Recently, spontaneous miniature hyperpolarizations (SMHs) have been reported in DA neurons of neonatal rats using intracellular recordings in brain slices (Seutin et al 1998). SMHs are dependent on internal Ca^{2+} mobilization and SK channel activation (Seutin et al 2000). In a series of experiments trying to elucidate the triggering mechanism and the functional significance of these SMHs, we found that spontaneous miniature outward currents (SMOCs; Fig. 3.9), which mediate the SMHs recorded with intracellular recordings, were solely mediated by Ca^{2+} release from intracellular stores via

RyRs. SMOCs were eliminated by depleting Ca^{2+} stores with CPA (10 μM; Fig. 3.10A) or ryanodine (20 μM; Fig. 3.10B), and by intracellular application of RyR antagonist ruthenium red (100 μM; Fig 3.10C), but not by IP_3R antagonist heparin (1mg/ml; Fig 3.10C2). Thus SOMCs can be used as a tool to study the effect of PI-coupled receptor activation on RyR-mediated Ca^{2+} release.

In line with the previous study that amphetamine-induced α1AR activation inhibited mGluR-mediated Ca^{2+} release from intracellular stores (Paladini et al 2001), we tested the effect of amphetamine on SMOCs. Bath application of amphetamine (10 μM) reduced the SMOC frequency to 44 ± 6% of control (n = 9). This inhibition was reversed by co-application of prazosin (100 nM), an α1AR antagonist (n = 4; Fig. 3.11A,B), but not by eticlopride (200 nM), a D2 DA receptor antagonist (n = 5). Similar inhibition was also observed with bath perfusion of selective α1AR agonist phenylephrine (30 μM; 61 ± 6% of control, n = 5; Fig. 3.11C) and DA (100 μM; 57 ± 5% of control, n = 4; Fig. 3.11D). All together, these results indicate that tonic activation of α1ARs inhibits Ca^{2+} release mediated via RyRs.

3.2.2.2 Effects of PI-coupled receptor activation on IP_3 concentration-response (C-R) curve

We further obtained evidence supporting the store depletion hypothesis by testing the effect of IP-coupled receptor activation on IP_3 C-R curve. The rationale is that if PI-coupled receptor agonists only cause desensitization of

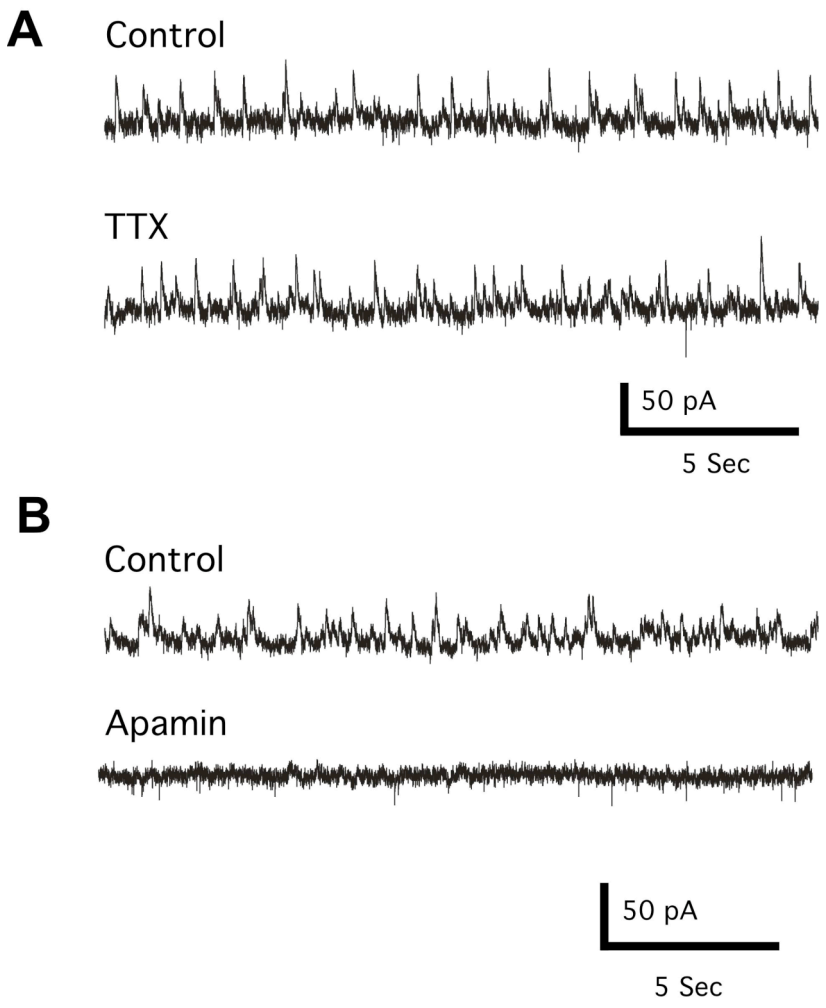

Figure 3.9. Spontaneous miniature outward currents (SMOCs) in DA neurons of neonatal rats.

(A) SMOCs are insensitive to TTX (200 nM – 1 μM). (B) SMOCs are blocked by superfusion of SK channel blocker apamin.

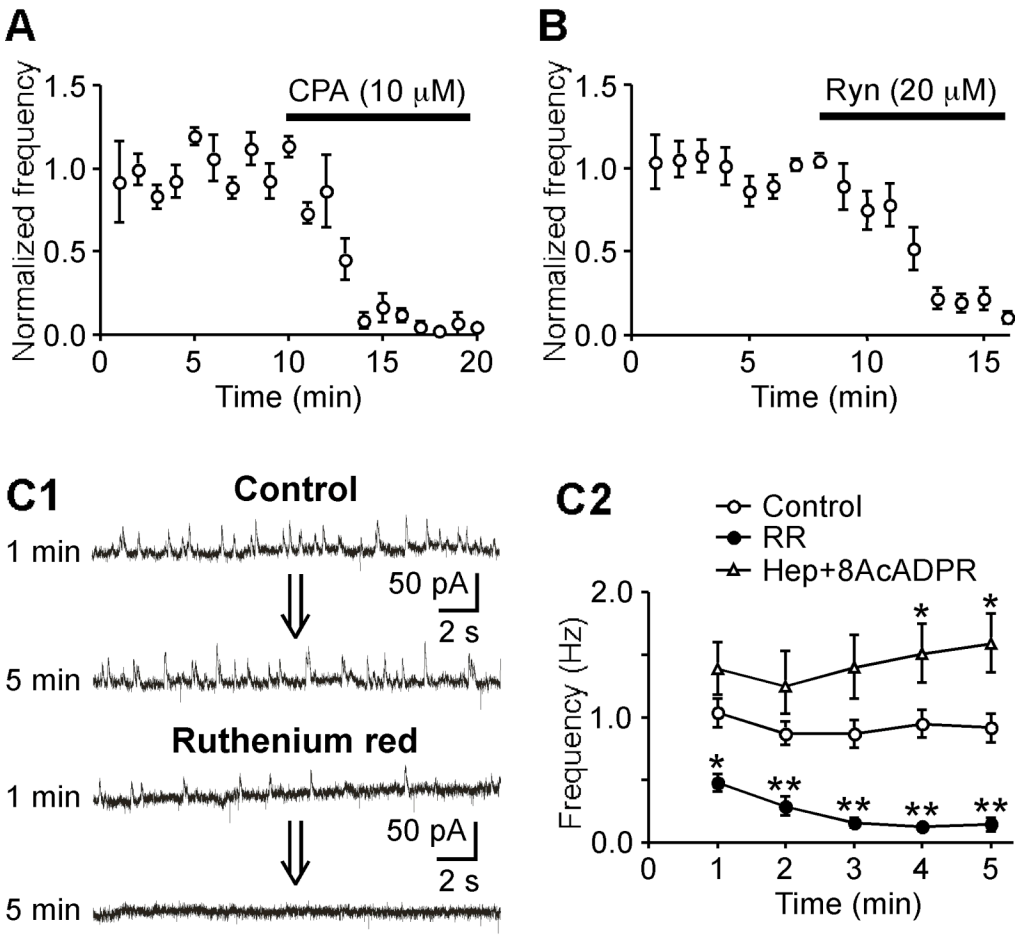

Figure 3.10. SMOCs are dependent on Ca^{2+} release from internal stores through RyRs.

(A) Summary time graph showing the effect of CPA (10 μM) on SMOCs (n = 4). (B) summary time graph illustrating the effect of ryanodine (Ryn; 20 μM) on SMOCs (n = 6). Both CPA and ryanodine eliminated SMOCs in ~5 min. (C1) Representative traces of SMOCs with a control internal solution or with a solution containing ruthenium red (100 μM). Traces are shown at 1 and 5 min after entering the whole-cell configuration. (C2) The SMOC frequency is plotted against time after going into the whole-cell configuration. Recordings were made with a pipette containing a control internal solution (n = 22), ruthenium red (RR; 100 μM, n = 11), and both heparin (Hep; 1 mg/ml) and 8-NH_2-cADPR (8AcADPR; 50 μM) (n = 8). Only ruthenium red induced significant inhibition of SMOCs. *$p < 0.05$, **$p < 0.01$ vs control.

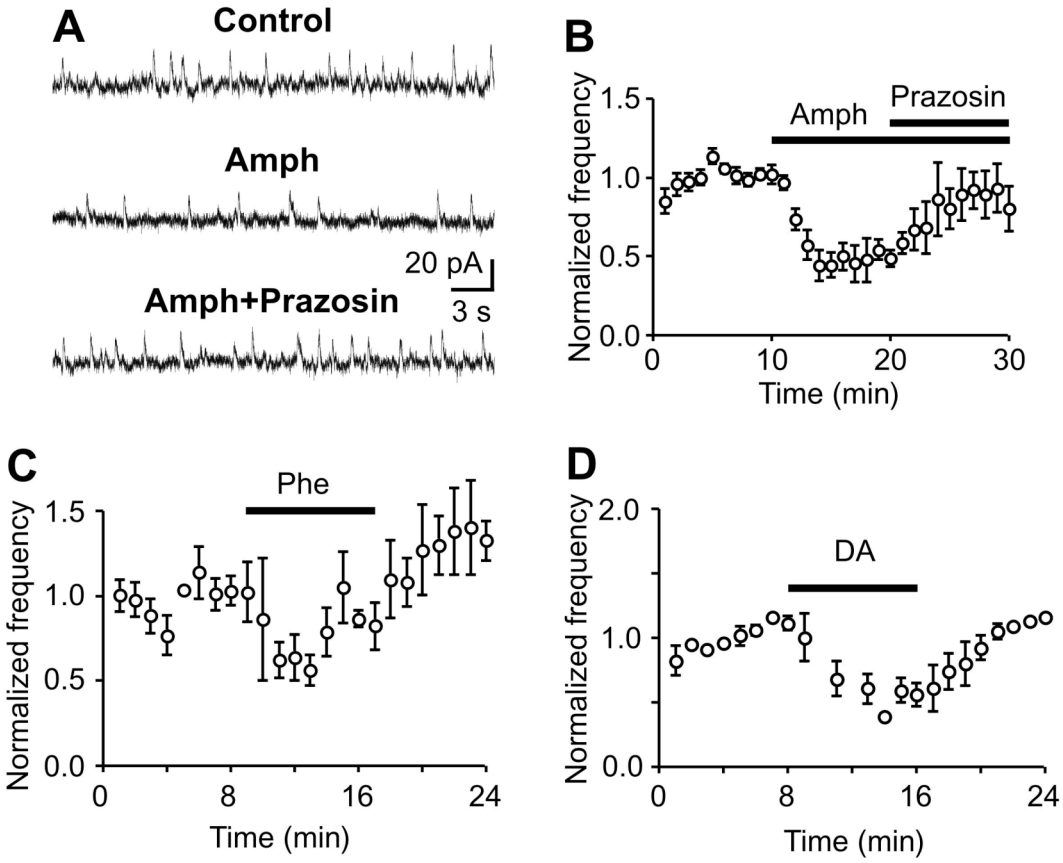

Figure 3.11. Amphetamine suppresses SMOCs via activation of α1 adrenergic receptors (α1ARs).

(A) Representative traces of SMOCs in a control extracellular solution, amphetamine (Amph; 10 μM), and amphetamine plus prazosin (100 nM). (B) A summary time graph illustrating the effect of amphetamine on SMOCs and its reversal by prazosin (n = 4). (C) and (D) Summary time graphs showing the effect of phenylephrene (Phe), a selective α1AR agonist, and DA on SMOCs.

IP_3Rs, there should a right shift of the IP_3 C-R curve. The maximal response induced by saturating concentration of IP_3 should not be affected (as illustrated in Fig. 3.12A2).

To plot an IP_3 C-R curve, we performed flash photolysis of caged IP_3 using different intensities of UV pulse, which determine the concentrations of IP_3 released (see Experimental Procedures). Photo-released IP_3 induces IP_3R-mediated intracellular Ca^{2+} mobilization and an SK-mediated outward current (I_{IP3}) in DA neurons (Fig. 3.12A1) (Morikawa et al 2000). To examine the effect of PI-coupled receptor activation on IP_3 C-R curve, we measured I_{IP3} evoked by two intensities of UV pulses: the intensity that produced ~50% (EC_{50}) of the maximal current amplitude and a supra-maximal intensity (Max) before and during bath application of DHPG (1 μM). DHPG induced comparable inhibition of I_{IP3} evoked by EC_{50} and saturating IP_3 (Fig. 3.12B). This result further demonstrated that PI-coupled receptor activation-induced increase in IP_3 tone inhibits IP_3R-mediated Ca^{2+} by causing partial depletion of Ca^{2+} stores (Verkhratsky 2005).

3.2.3 PI-COUPLED RECEPTOR ACTIVATION AUGMENTS I_{AHP} VIA FACILITATING CICR

Previous studies using lipid bi-layers have demonstrated that cytosolic IP_3 can dose-dependently facilitate IP_3R-mediated CICR (Bezprozvanny et al 1991).

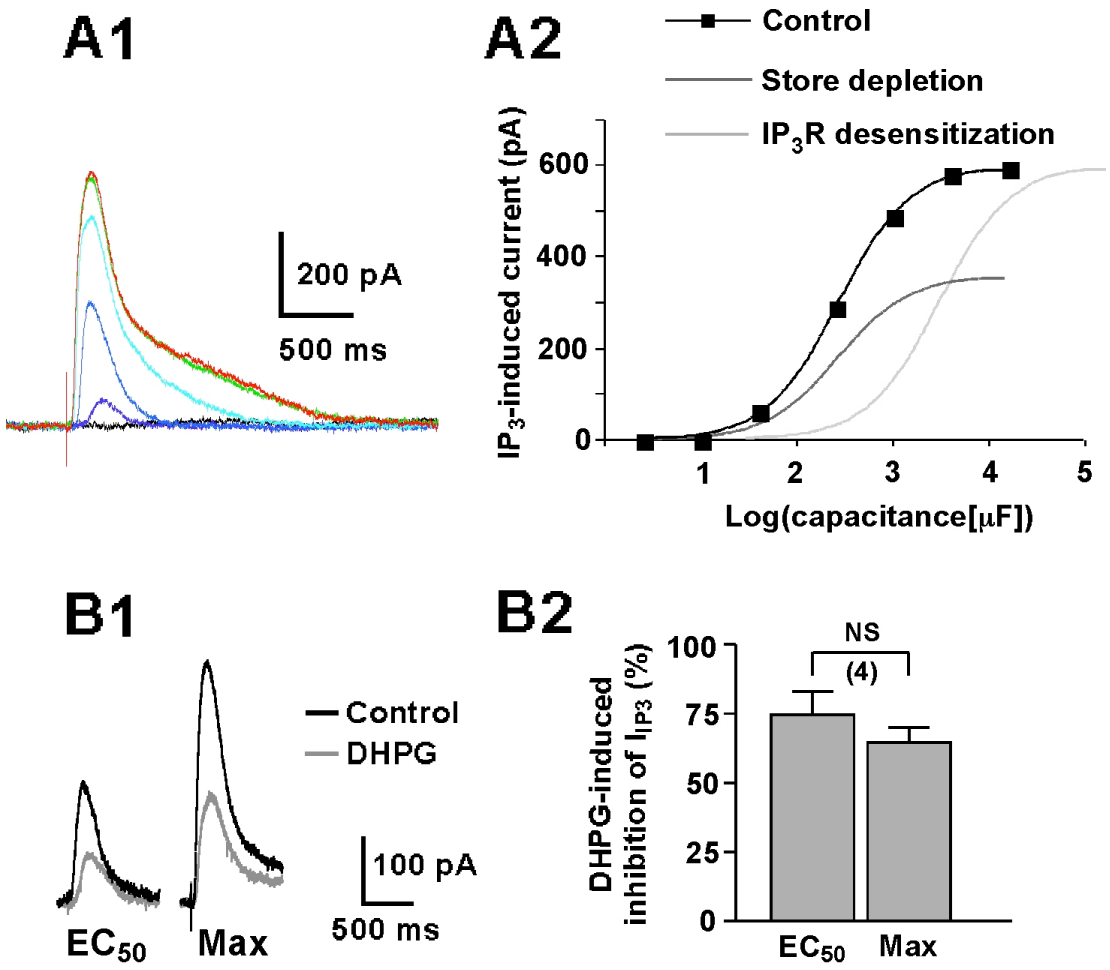

Figure 3.12. Effect of DHPG on IP_3 concentration-response (C-R) curve.

(A1) Representative traces of outward currents induced by flash photolysis of caged IP_3 with UV pulses of different intensities. The intensity of UV pulse was controlled by the capacitance of the UV generating device. The selected capacitances in (A1) were 50, 100, 200, 500, 1000, 2000, and 4000 μF. UV pulses of 50 and100 μF did not induce visible currents in this neuron. (A2) The black curve shows IP_3 C-R curve plotted from the result in (A1). Dark grey and light grey curves are theoretical curves in the cases of Ca^{2+} store depletion and IP_3R desensitization, respectively. (B1) Representative traces of IP_3-induced outward currents evoked by a UV pulse that produced 50% of maximal current (EC_{50}), and a supra-maximal UV pulse (Max). Note that DHPG produced comparable inhibition on currents evoked by EC_{50} intensity and Max intensity. (B2) Summary bar graph showing that DHPG induced similar magnitude of inhibition on IP_3-currents evoked by EC_{50} and Max intensities of UV flash.

To examine whether PI-coupled receptor-induced facilitation of AP-induced Ca^{2+} signals is attained via enhancing CICR from intracellular stores, we first used CPA to deplete these stores. The effects of DHPG (1 μM, n = 10) and phenylephrine (10 μM, n = 4) on I_{AHP} were completely abolished by CPA (10-20 μM) (Fig. 3.13). Furthermore, the outward current produced by flash photolysis of caged Ca^{2+} ($I_{caged\text{-}Ca}$), which directly liberates Ca^{2+} inside the cell, was also increased by DHPG and phenylephrine in a CPA-sensitive fashion (Fig. 3.14). Although CPA itself appeared to irreversibly suppress $I_{caged\text{-}Ca}$, we did not quantify this effect because $I_{caged\text{-}Ca}$ showed significant rundown over time. Thus, our results indicate that tonic activation of PI-coupled receptors augments AP-induced Ca^{2+} signals by facilitating CICR.

3.2.4 Intracellular photolytic application of IP_3 reconstitutes PI-coupled receptor-induced differential effects on I_{AHP} and I_{mGluR}

To directly demonstrate the role of intracellular IP_3 tone, we next performed flash photolysis of caged IP_3 to elevate intracellular IP_3 concentration. We used a single UV pulse of near-threshold intensity, which barely produced an outward current by itself, to release IP_3 50 ms before evoking I_{AHP}. IP_3 dramatically facilitated I_{AHP} in a manner similar to perfusion of PI-coupled receptor agonists (n = 5; Fig. 3.15A). Furthermore, I_{mGluR} was inhibited by repetitive photolytic applications of IP_3 at threshold intensity for 3 s, which would

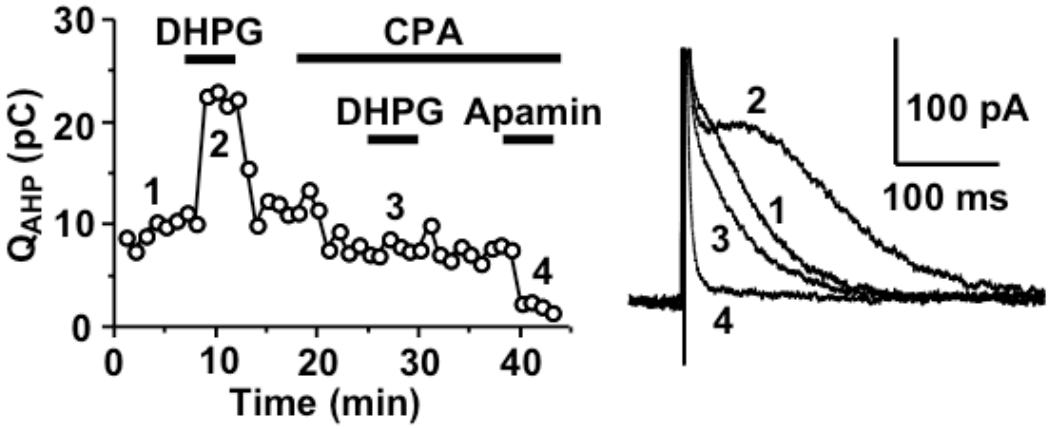

Figure 3.13. DHPG-induced facilitation of I_{AHP} is Ca^{2+} store-dependent.

Time graph (left) and the corresponding traces (right) from a single recording illustrating that the facilitatory effect of DHPG (1 μM) on I_{AHP} was blocked by CPA (10 μM).

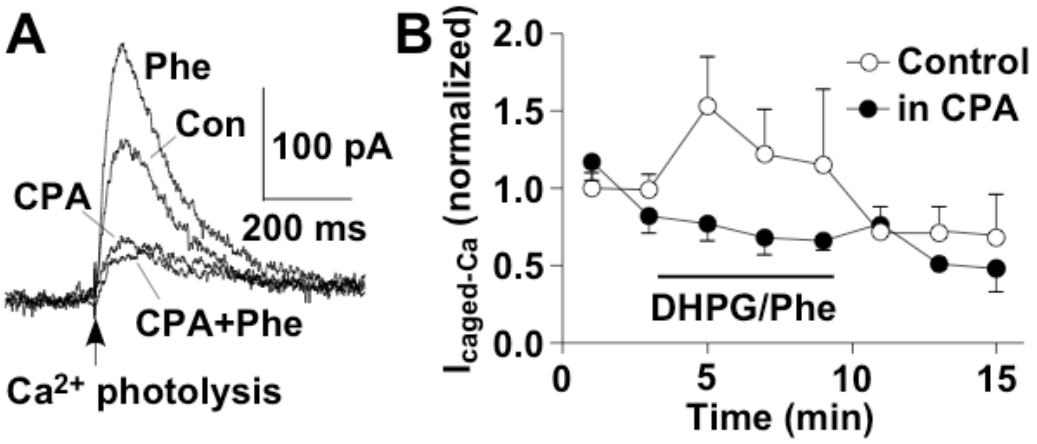

Figure 3.14. DHPG enhances the outward current ($I_{caged\text{-}Ca}$) induced by flash photolysis of caged Ca^{2+} inside the cell.

(A) Representative traces of $I_{caged\text{-}Ca}$ depicting the facilitatory effect of phenylephrine (Phe, 10 μM) and its CPA-sensitivity. Photolytic release of Ca^{2+} was made at the time indicated by the arrow. (B) Summary time graph showing that DHPG (1 μM) and Phe augmented $I_{caged\text{-}Ca}$ in a CPA-dependent fashion.

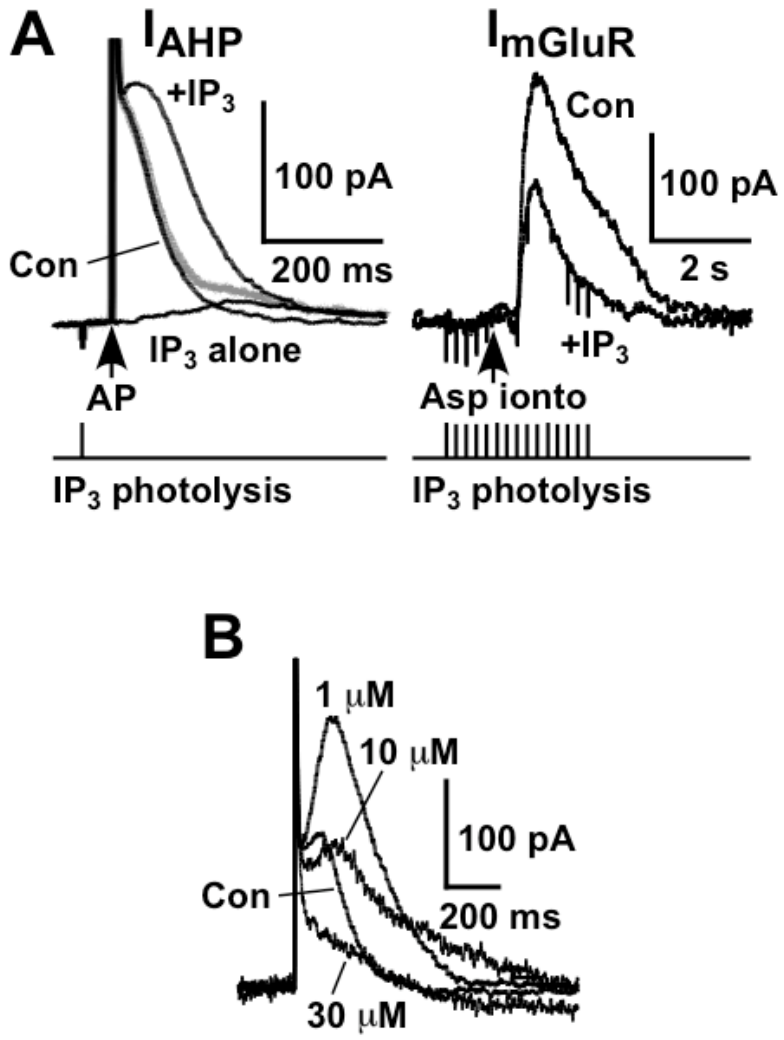

Figure 3.15. IP_3 mediates differential regulation of AP- and phasic mGluR-induced Ca^{2+} signals.

(A) Left: Representative traces of I_{AHP} with and without photolytic release of IP_3. UV flash was applied 50 ms prior to the 2-ms depolarizing pulse (arrow). The current elicited by IP_3 without the depolarizing pulse is also shown. The gray trace represents simple summation of control I_{AHP} and the current evoked by IP_3 alone. Right: Representative traces of I_{mGluR} with and without repetitive photolytic release of IP_3. Aspartate iontophoresis (200 ms) was made at the time indicated by the arrow. UV flash was applied at 5 Hz for 3 s, starting 1 s prior to aspartate iontophoresis. (C) Representative traces of I_{AHP} illustrating biphasic effects of DHPG at different concentrations.

gradually deplete Ca^{2+} stores (n = 4; Fig. 3.15A). Therefore, a small increase in cytosolic IP_3 concentration reproduced the regulation of I_{AHP} and I_{mGluR} induced by sustained activation of PI-coupled receptors. A higher concentration of DHPG (30 μM) caused inhibition of I_{AHP} instead of facilitation in 5 out of 8 cells tested (Fig. 3.15B), suggesting that high IP_3 levels produced by strong activation of mGluRs more fully deplete Ca^{2+} stores and thus can suppress CICR triggered by APs.

3.2.5 ACTIVITY-DEPENDENT REGULATION OF AP-INDUCED CA^{2+} SIGNALS

DA neurons tonicly fire APs at 1-5 Hz and also display phasic bursts comprising 2-10 spikes at 10-50 Hz *in vivo* (Kitai et al 1999; Overton & Clark 1997). To investigate the regulation of AP-induced Ca^{2+} signals under physiological conditions, we evoked I_{AHP} in a train of 5 test pulses at 2 Hz to mimic the tonic firing pattern, and studied effect of tonic firing on single AP-and burst-induced Ca^{2+} responses.

3.2.5.1 Effect of tonic firing on single AP-induced I_{AHP}

When evoked in a 2-Hz train, Q_{AHP} was rapidly reduced (Fig. 3.16A), from 17.3 ± 2.1 pC for the first I_{AHP} (Q_{AHP-1}) to 11.2 ± 2.0 pC for the fifth one (Q_{AHP-5}) (n = 7, $p < 0.01$; Fig. 3.16B). The reduction in Q_{AHP} during the 2-Hz train was largely eliminated in the presence of CPA (9.3 ± 2.5 pC for Q_{AHP-1} vs. 8.2 ± 1.9 pC for Q_{AHP-5}, n = 5, $p > 0.05$). Although CPA still produced inhibition of Q_{AHP-5} in

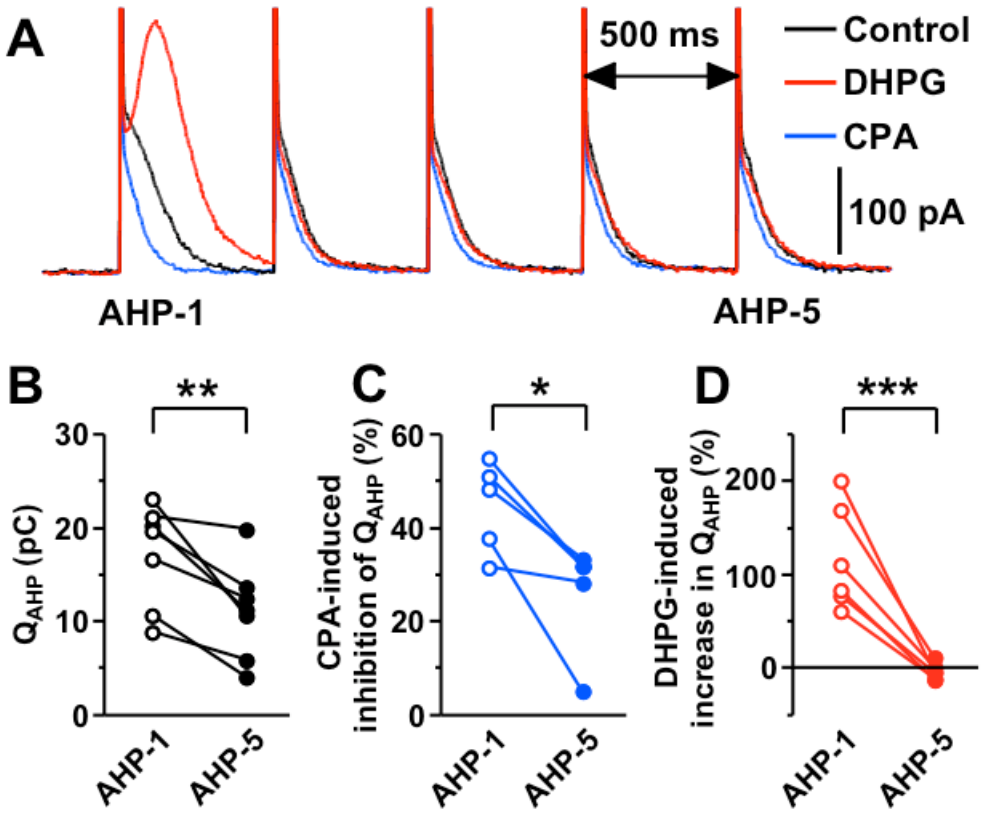

Figure 3.16. Repetitive APs at 2 Hz depress single AP-induced CICR.

(A) Representative traces of I_{AHP} evoked by a train of 5 test pulses at 2 Hz in control (*black*), DHPG (1 μM; *red*), and CPA (10 μM; *blue*). (B-D) Q_{AHP}, CPA-induced inhibition of Q_{AHP}, and DHPG-induced increase in Q_{AHP} for the first (AHP-1) and fifth (AHP-5) test pulse in the 2-Hz train are plotted for each cell. Note that DHPG had no facilitating effect on AHP-5.

the 2-Hz train, the magnitude of inhibition was significantly smaller compared to that of Q_{AHP-1} (45 ± 4% inhibition for Q_{AHP-1} vs. 26 ± 5% inhibition for Q_{AHP-5}, n=5, $p < 0.05$; Fig. 3.16C). Therefore, AP-induced CICR was attenuated with repetitive APs at 2 Hz, causing a reduction in Q_{AHP}.

3.2.5.2 Tonic firing induces inactivation of IP_3Rs

Surprisingly, the facilitatory effect of DHPG (1 μM) on I_{AHP} was abolished with the 2-Hz AP train (118 ± 23% increase for Q_{AHP-1} vs. -2 ± 4% increase for Q_{AHP-5}, n = 6, $p < 0.001$; Fig. 3.16A,D). Consistent with this, the facilitatory effect of IP_3, photolytically applied at threshold UV intensity, was also significantly depressed by the AP train (n = 6, $p < 0.05$; Fig. 3.17A,B). The suppression of IP_3 facilitation of CICR may be due to inactivation, or reduced sensitivity, of IP_3Rs caused by $[Ca^{2+}]_i$ elevations during the AP train (Taylor and Laude, 2002). Alternatively, AP-induced CICR may reduce the size of Ca^{2+} stores to prevent further CICR. Indeed, depolarization-induced Ca^{2+} influx can either increase or reduce the Ca^{2+} store size depending on the relative rates of Ca^{2+} uptake and release (Albrecht et al., 2001). In order to address these possibilities, we first asked if the AP train alters the IP_3 sensitivity of IP_3Rs. Here, we performed flash photolysis of caged IP_3 at 500 ms after the 2-Hz AP train using different intensities of UV pulse, thereby varying the concentrations of IP_3 released. The AP train had no effect on the IP_3-mediated outward current (I_{IP3}) evoked with a supra-maximal UV intensity. However, the same AP train caused significant depression of I_{IP3} elicited using lower UV intensities that produced ~20% (EC_{20})

or ~50% (EC_{50}) of the maximal current amplitude (Fig. 3.17C). The magnitude of AP train-induced reduction in I_{IP3} became smaller with an increase in the UV intensity, i.e., with an increase in the IP_3 concentration (Fig. 3.17D). We also found that a UV intensity that was subthreshold when applied 500 ms after the AP train was able to evoke a measurable outward current when applied without the AP train, indicating that the AP train elevated the threshold for evoking I_{IP3} (n = 3, data not shown). In contrast, bath application of DHPG (1 μM) produced comparable inhibition of I_{IP3} evoked by UV pulses at EC_{50} and supra-maximal intensities (Fig. 3.12B). These data are consistent with the idea that: 1) repetitive APs cause inactivation of IP_3Rs by reducing their IP_3 sensitivity, and 2) DHPG-induced increase in IP_3 tone inhibits IP_3R-mediated Ca^{2+} release with no change in IP_3 sensitivity, as would be expected if the driving force for Ca^{2+} release is diminished by a reduction in the store Ca^{2+} concentration. Furthermore, the reduction in I_{IP3} and Q_{AHP} caused by the 2-Hz AP train recovered over a period of several seconds (Fig. 3.17E,F), in good agreement with the recovery kinetics of Ca^{2+}-dependent inactivation of IP_3Rs (Parker and Ivorra, 1990; Finch et al., 1991), but much faster than the time course of Ca^{2+} store replenishment that takes place over minutes (Albrecht et al., 2001; Solovyova and Verkhratsky, 2003). Altogether, these results strongly suggest that repetitive AP-induced Ca^{2+} influx produces inactivation of IP_3Rs during the AP train.

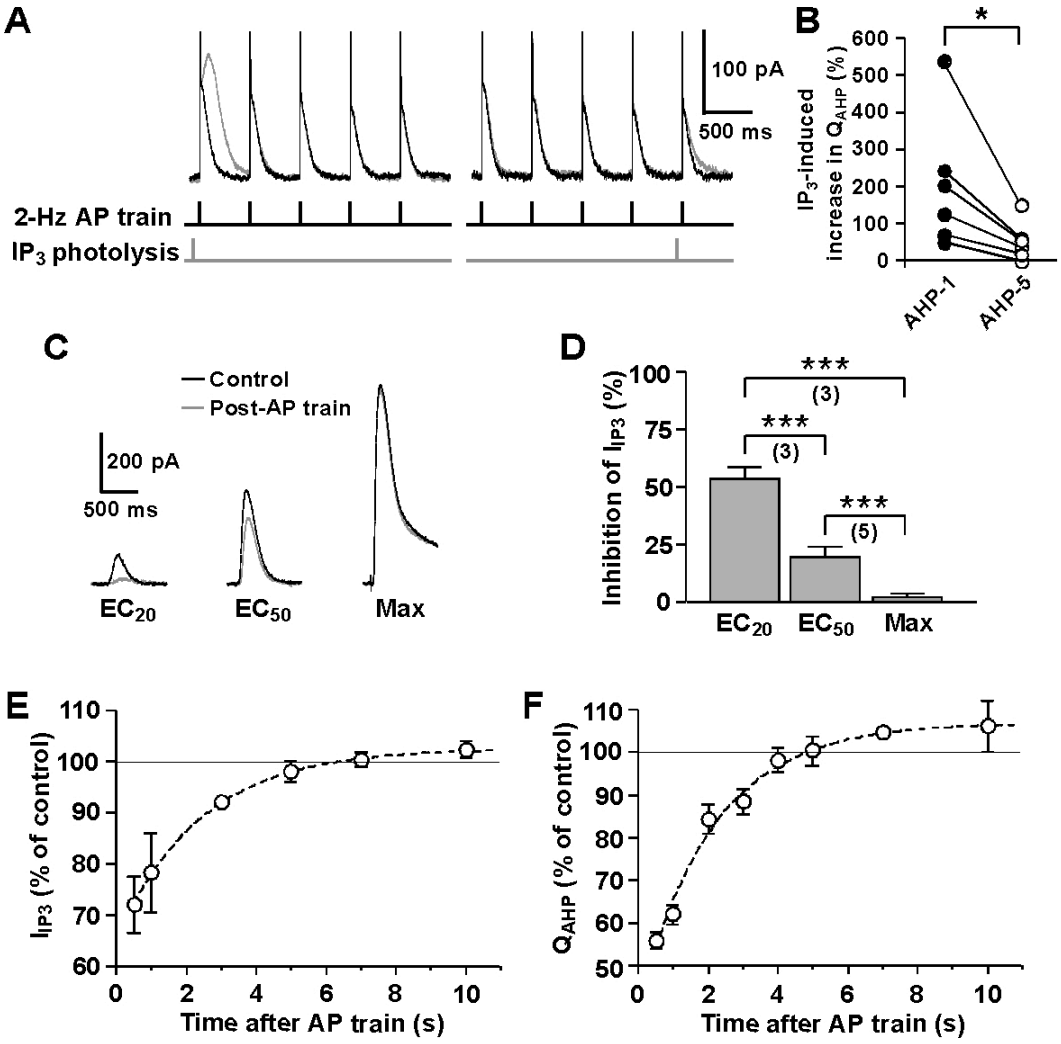

Figure 3.17. Repetitive APs at 2 Hz induce IP_3R inactivation.

(A) Traces of I_{AHP} evoked by a train of 5 test pulses at 2 Hz with (gray) and without (black) photolytic release of IP_3. UV flash was applied 50 ms prior to the first (left) or fifth (right) test pulse. (B) The magnitude of increase in Q_{AHP} by photolytic release of IP_3 at threshold intensity for the first (AHP-1) and fifth (AHP-5) test pulses in the AP train is plotted for each cell. (C) Representative traces of I_{IP3} with (gray) and without (black) a preceding 2-Hz train of 5 depolarizing pulses. UV flash was applied 500 ms after the fifth pulse of the train. The intensity of UV flash was varied to elicit maximal current amplitude (Max) and ~20% (EC_{20}) or ~50% (EC_{50}) of maximal current. (D) Summary bar graph showing the magnitude of inhibition of I_{IP3}, evoked with different UV intensities, by a 2-Hz AP train. Note that the 2-Hz train produced larger inhibition as the UV intensity was reduced. (E,F) The recovery time course of I_{IP3} (n = 3; E) and Q_{AHP} (n = 5; F) after the 2-Hz AP train. The intensity of UV flash was ~EC_{50} for the experiments in E. The dotted lines represent single exponential fit to the data. *$p < 0.05$, ***$p < 0.001$.

3.2.5.3 Effect of tonic firing on Burst-induced Ca^{2+} signals

We further investigated the influence of 2-Hz AP train on burst-induced Ca^{2+} signals. Burst was elicited by a train of 5 test pulses at 20 Hz. As expected, $Q_{AHP\text{-}burst}$ was significantly reduced when the burst was preceded by a 2-Hz train compared to the one elicited in isolation (64.3 ± 8.4 pC for isolated burst vs. 47.0 ± 5.3 pC for post-2 Hz burst, n = 6, $p < 0.05$; Fig. 3.18A,B). In line with this, the 2-Hz train caused 33 ± 5 % reduction in burst-induced fluorescence change at proximal dendrites (~20-50 μm from the soma), using fluo-5F (50 μM) or fluo-4FF (100 μM; K_d = 9.7 μM) as Ca^{2+} indicators (n = 6, 4 cells in voltage clamp and 2 cells in current clamp; Fig. 3.18E,F). Furthermore, the effect of CPA on $Q_{AHP\text{-}burst}$ was attenuated by the AP train (38 ± 4% inhibition for the isolated burst vs. 32 ± 4% inhibition for the post-AP train burst, n = 5, $p < 0.05$; Fig. 3.18C), consistent with a reduction in CICR. However, DHPG, which had no effect on $Q_{AHP\text{-}5}$ in the AP train (Fig. 3.16A,D), produced a significant increase in $Q_{AHP\text{-}burst}$ following the AP train (30 ± 9% increase, n = 6; 3.18A,D), although the magnitude of increase was smaller compared to that for the isolated burst (53 ± 12% increase, $p < 0.01$).

Taken together, these results suggest that tonic firing inhibits both single AP- and burst-induced Ca^{2+} signals via inactivating IP_3Rs. PI-coupled receptor activation-induced rise in cytosolic IP_3 selectively augments large Ca^{2+} influx associated with bursts.

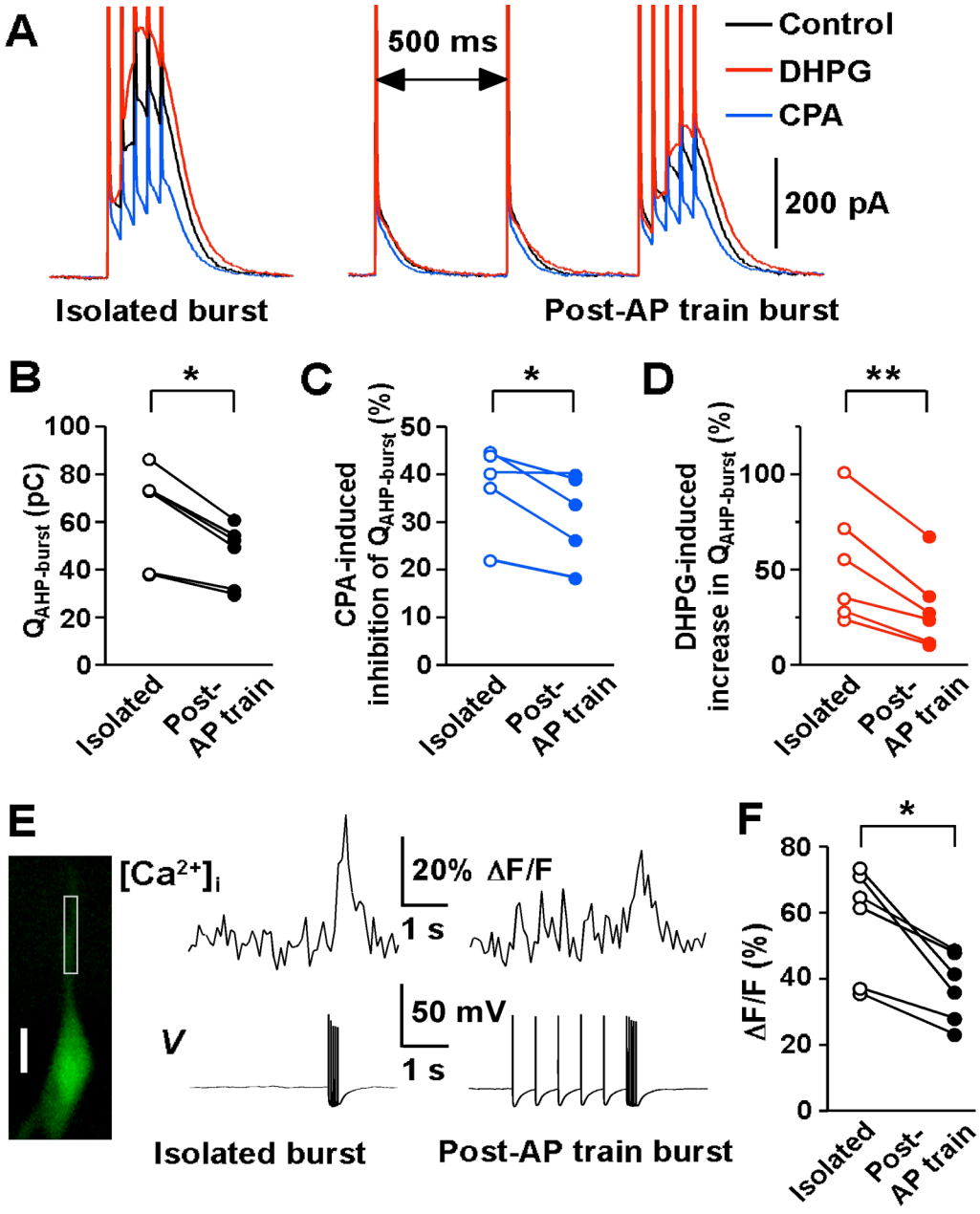

Figure 3.18. Repetitive APs at 2 Hz depress burst-induced CICR.

(A) Representative traces of outward currents ($I_{AHP\text{-}burst}$) evoked by a train of 5 test pulses at 20 Hz (burst) in control (black), DHPG (red), and CPA (blue). A 20-Hz burst was elicited alone (isolated burst; left) or 500 ms after a 2-Hz, 5-pulse train (post-2 Hz burst; right). The first three I_{AHP}s in the 2-Hz train are not shown. (B-D) $Q_{AHP\text{-}burst}$, CPA-induced inhibition of $Q_{AHP\text{-}burst}$, and DHPG-induced increase in $Q_{AHP\text{-}burst}$ for isolated burst and post-2 Hz burst are plotted for each cell. (E) A current clamp recording showing that evoking APs at 2 Hz suppressed burst-induced Ca^{2+} transient. A confocal fluorescence image of a DA neuron loaded with fluo-5F (50 μM) is shown on the left (scale bar = 20 μm). A 20-Hz burst was elicited alone or following a 2-Hz, 5-pulse train. Fluorescence changes were measured at the ROI indicated, while the membrane potential was recorded in the same cell (bottom traces). (F) Fluorescence changes induced by the isolated burst and the post-AP train burst are plotted for each cell. *$p < 0.05$, **$p < 0.01$.

3.3 Aim 3: Effect of amphetamine on burst-induced Ca^{2+} signals

The findings above also imply that suppression of basal firing can remove IP_3R inactivation and increase the effect of IP_3 on burst-evoked Ca^{2+} signals. It is well known that psychostimulant amphetamine causes somatodendritic release of DA and suppresses tonic DA neuron firing via D2 autoreceptor (D2R) activation (Mercuri et al 1989), an effect that should increase burst-induced Ca^{2+} signals and their IP_3-mediated facilitation. However, it is not clear how amphetamine affects the burst firing itself. To address this issue, we performed cell-attached recordings of DA neuron firing.

3.3.1 AMPHETAMINE SELECTIVELY INHIBITS BASAL FIRING

Iontophoretic application of aspartate elicited an iGluR-mediated burst followed by an mGluR-mediated pause, as reported previously (Morikawa et al 2003). Bath application of amphetamine (10 μM) dramatically decreased the basal firing rate by 86 ± 3% (from 2.4 ± 0.2 Hz to 0.3 ± 0.1 Hz, n = 5; Fig. 3.19A,B). On the other hand, amphetamine produced a relatively small inhibition of the firing rate within the burst (27 ± 4% inhibition, from 19.0 ± 3.1 Hz to 13.8 ± 2.3 Hz) and the number of spikes per burst (26 ± 3% inhibition, from 7.4 ± 0.6 to 5.4 ± 0.3). Hence, amphetamine relatively spared burst firing compared to its massive inhibition of tonic firing.

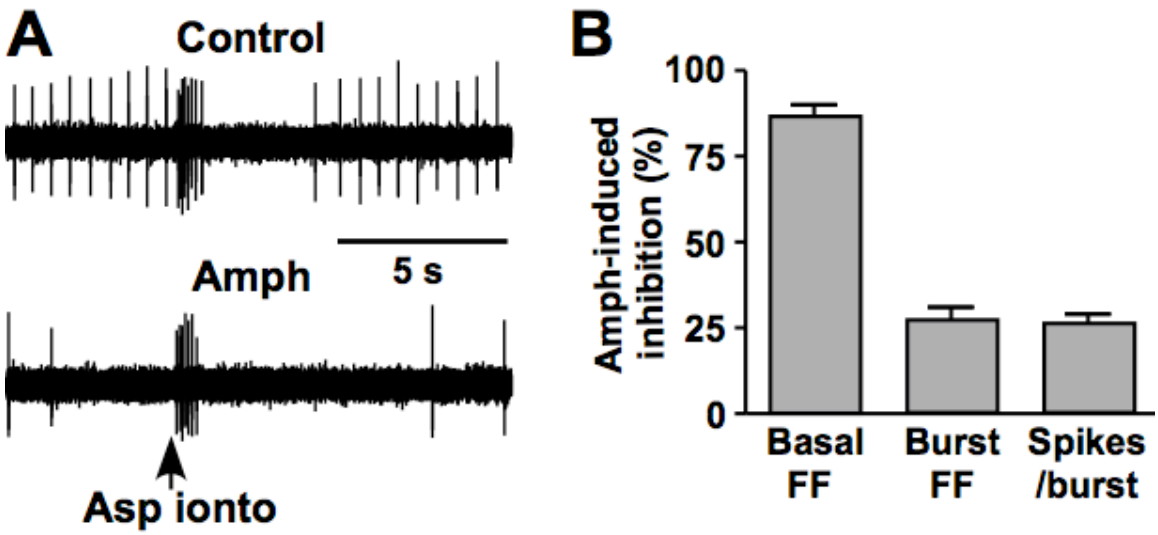

Figure 3.19. Amphetamine selectively inhibits basal firing.

(A) Representative traces of DA neuron firing in control and in amphetamine (Amph; 10 μM) recorded with a cell-attached configuration. The burst was elicited by iontophoretic application of aspartate (50 ms). (B) Summary bar graph illustrating that amphetamine dramatically depressed basal firing rate (FR) with relatively small effects on the burst. The data are from 5 cells.

3.3.2: POTENTIAL DUAL MECHANISMS OF AMPHETAMINE ACTION ON BURST-EVOKED Ca^{2+} SIGNALS

Amphetamine-induced DA release can also activate α1ARs in DA neurons (Cui et al 2004; Paladini et al 2001). Therefore, it is possible that amphetamine augments burst-induced Ca^{2+} signals by D2R-mediated inhibition of tonic firing and α1AR-mediated facilitation of CICR. To test this possibility, we first examined the effect of amphetamine-induced firing pattern changes on burst-evoked Ca^{2+} signals. In these experiments, I_{AHP} was evoked using two different protocols: 1) a control protocol simulating the firing pattern in control condition (7 test pulses at 19 Hz for burst, preceded by 5 test pulses at 2.4 Hz for basal firing) and 2) an amphetamine protocol mimicking the firing pattern in amphetamine (5 test pulses at 14 Hz for burst, preceded by 2 test pulses at 0.3 Hz for basal firing) (Fig. 3.20A). $Q_{AHP\text{-}burst}$ induced with the amphetamine protocol was significantly larger than that elicited with control protocol (44 ± 11% increase, n = 11; Fig. 3.20A,B). Phenylephrine (10 μM) superfused while evoking I_{AHP} with amphetamine protocol further increased $Q_{AHP\text{-}burst}$ by 17 ± 5% (n = 5). These results suggest that amphetamine can augment burst-induced Ca^{2+} signals by D2R-mediated suppression of tonic AP firing and by α1AR-mediated facilitation of CICR.

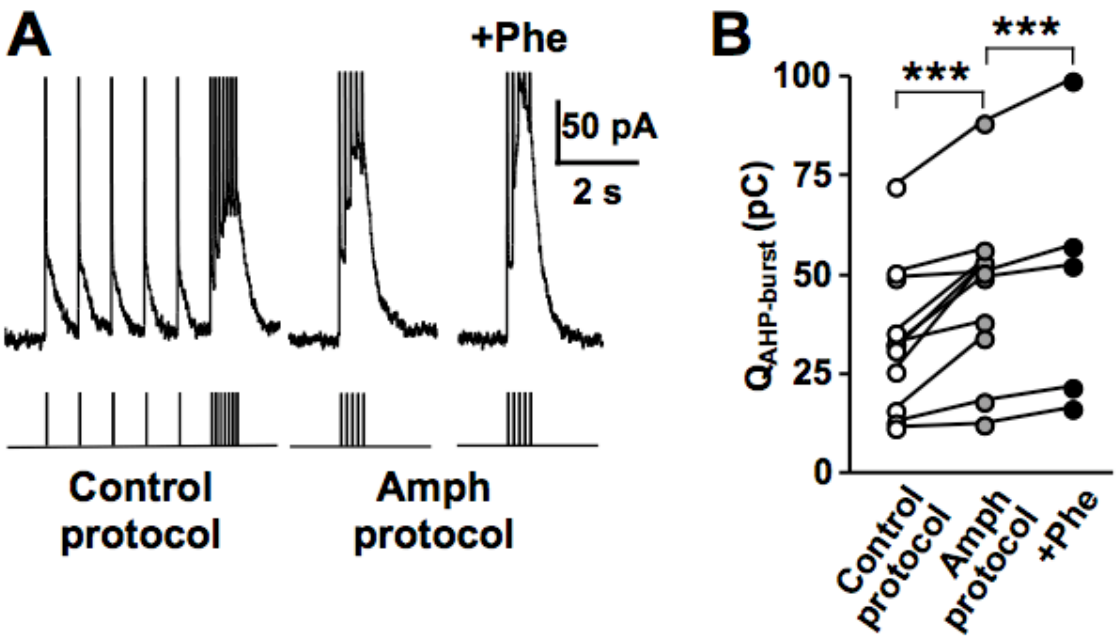

Figure 3.20. Dual mechanisms for amphetamine to augment the burst-evoked Ca^{2+} signals.

(A) Representative traces of $I_{AHP\text{-}burst}$ generated by a protocol simulating the control firing pattern (19-Hz, 7-AP burst preceded by 5 APs at 2.4 Hz; left) and by a protocol mimicking the firing pattern under the impact of amphetamine (14-Hz, 5-AP burst preceded by 2 APs at 0.33 Hz; middle. The 2 APs at 0.33 Hz are not shown). A trace of $I_{AHP\text{-}burst}$ evoked by the amphetamine protocol in the presence of phenylephrine (10 μM) is also shown on the right. All three traces are from the same cell. (B) $Q_{AHP\text{-}burst}$ of individual cells recorded in three different conditions as in (A). Switching from the control protocol to the amphetamine protocol induced a significant increase in $Q_{AHP\text{-}burst}$, which was further augmented by phenylephrine. ***$p < 0.001$.

CHAPTER 4: DISCUSSION

By measuring Ca^{2+}-sensitive SK conductance and fluorescence of Ca^{2+} indicator dyes in DA neurons, we have demonstrated a unique mechanism to differentially regulate two functionally and mechanistically distinct Ca^{2+} signals, where tonic activation of PI-coupled metabotropic receptors enhances AP-evoked Ca^{2+} transients while inhibiting the Ca^{2+} signal caused by phasic mGluR activation. Both of these effects were mediated by a small rise in intracellular IP_3 tone, which caused 1) sensitization of IP_3Rs to Ca^{2+}-dependent activation and 2) partial depletion of intracellular Ca^{2+} stores. This differential regulation was further tuned by the context in which APs were generated, in a manner that selectively amplifies burst-induced Ca^{2+} signals in tonicly firing DA neurons. Therefore, Ca^{2+} signals triggered by phasic bursts may play a privileged role when DA neurons receive tonic neurotransmitter inputs generating IP_3. Furthermore, we showed that amphetamine selectively inhibited tonic firing. This effect, together with amphetamine-induced activation of α1ARs, augmented burst-induced Ca^{2+} signals in DA neurons.

4.1 IP_3 amplifies AP-induced CICR

Our results show that APs trigger CICR through both IP_3Rs and RyRs under resting conditions. An intriguing characteristic of IP_3Rs and RyRs is that they are each synergistically coactivated by Ca^{2+} and another intracellular

messenger; IP_3 for IP_3Rs and cADPR for RyRs (Berridge 1998). Accordingly, a rise in IP_3 or cADPR levels can augment AP-induced CICR via IP_3Rs or RyRs (Hua et al 1994; Nakamura et al 2000). Previous studies have shown that both IP_3 and cADPR contribute to Ca^{2+} release produced by strong, transient (50-200 ms) activation of mGluRs and α1ARs in DA neurons (Morikawa et al 2003; Paladini & Williams 2004). However, only the IP_3 pathway was involved in the facilitation of CICR by relatively weak, tonic activation of these receptors, attained either by bath perfusion of agonists or by sustained (~1 s) stimulation of presynaptic fibers at an intensity subthreshold for Ca^{2+} wave generation. It is likely that the cADPR concentration did not reach the level necessary to coactivate RyRs with Ca^{2+} under these conditions of weak receptor activation.

It has been shown that inhibiting the PLC-IP_3 pathway also suppresses the enhancement of AP-induced Ca^{2+} signals by mGluRs and mAChRs in hippocampal and cortical pyramidal neurons (Nakamura et al 2000; Power & Sah 2002; Yamada et al 2004) and the augmentation of SK-dependent AHPs by α1ARs in dorsal raphe neurons (Pan et al 1994). In these studies and ours, IP_3Rs are assumed to be the coincidence detector underlying the supralinear Ca^{2+} signal generated by concurrent metabotropic receptor activation and AP-induced Ca^{2+} influx. However, recent evidence in cerebellar Purkinje neurons indicates that PLC can be coactivated by G proteins and Ca^{2+}, resulting in supralinear production of IP_3 (Okubo et al 2004). Although this may be an interesting mechanism to pursue in DA neurons as well, our data showing that

direct photolytic application of IP_3, which bypasses G protein-PLC coupling, can reproduce the effect of receptor activation provide strong support for the major role of IP_3Rs as the coincidence detector.

Strong, focal activation of mGluRs elicits a slowly propagating Ca^{2+} wave in DA neurons as well as in hippocampal pyramidal neurons (Jaffe & Brown 1994; Morikawa et al 2003; Nakamura et al 1999), likely reflecting the diffusion of IP_3/cADPR or Ca^{2+}. In contrast, Ca^{2+} transients evoked by a burst of APs occurred simultaneously at the soma and proximal dendrites, which were enhanced by DHPG in terms of both the amplitude and duration. This observation suggests that IP_3 tone developed throughout the cell facilitated CICR triggered by rapidly propagating APs (Hausser et al 1995; Nakamura et al 1999). However, the amplitude of Ca^{2+} transients, as well as the magnitude of DHPG-induced enhancement, was larger in proximal dendrites than in the soma. Although the reason for this difference is uncertain, it is likely to result, at least partly, from the large volume of the soma diluting Ca^{2+} and/or IP_3 (Watanabe et al 2006; Wilson & Callaway 2000). Consistent with this idea, weak, sustained synaptic stimulation of mGluRs facilitated burst-induced Ca^{2+} signals only in the proximal dendrite close to the stimulating electrode but not in the soma or the opposite dendrite.

4.2 Firing pattern regulates IP_3R-mediated Ca^{2+} release

It is well established that IP_3Rs are biphasically regulated by cytosolic Ca^{2+} (Bezprozvanny et al 1991; Taylor & Laude 2002). Compared to rapid Ca^{2+}-dependent activation of IP_3Rs, inactivation induced by Ca^{2+} has a slow rate of onset (tens to hundreds of milliseconds) and lasts for seconds (Adkins & Taylor 1999; Finch et al 1991; Parker & Ivorra 1990). Action potentials induce Ca^{2+} influx through VGCCs and increase $[Ca^{2+}]_i$ (Jaffe et al 1994; Markram et al 1995). The increase in $[Ca^{2+}]_i$ correlates with the firing rate of the neuron (Abel et al 2004). Tonic firing of DA neurons produces repetitive and sustained elevation of $[Ca^{2+}]_i$ in the range of 100-200 nM (Wilson & Callaway 2000), which may well cause Ca^{2+}-dependent inactivation of IP_3Rs. Indeed, evoking APs repetitively at 2 Hz, mimicking the tonic firing, suppressed IP_3R-mediated CICR in this study. It should be noted that, during the 2-Hz AP train, IP_3-induced CICR was significantly diminished when the second AP was evoked, i.e., 500 ms after the first AP. Furthermore, this reduction mostly recovered after an AP-free interval of ~10 s (data not shown). These observations are in good agreement with the time course of Ca^{2+}-dependent inactivation of IP_3Rs described above. We further found that IP_3R inactivation caused by repetitive APs is associated with a decrease in the IP_3 sensitivity of IP_3Rs, consistent with previous studies

measuring single-channel activities of IP_3R channels inactivated by Ca^{2+} (Mak et al 1998; Moraru et al 1999).

It has been reported that IP_3Rs inactivated by a low concentration of Ca^{2+} (250 nM) can still be activated by a higher concentration of Ca^{2+} (1 μM) in the presence of a constant level of IP_3 (Finch et al 1991). In line with this, a large Ca^{2+} influx produced by a burst of APs was able to trigger CICR through IP_3Rs even when they were inactivated by repetitive APs. Importantly, this property enables tonic activation of PI-coupled receptors to selectively amplify burst-induced Ca^{2+} signals when DA neurons are constantly firing at low frequency.

In contrast to the reduction in IP_3R-mediated Ca^{2+} release caused by repetitive APs in this study, AP trains have been shown to augment mGluR-mediated Ca^{2+} release in DA neurons (Fiorillo & Williams 1998) and in hippocampal CA1 neurons (Jaffe & Brown 1994). This is likely due to the loading of Ca^{2+} stores resulting from AP-induced Ca^{2+} influx (Berridge 1998). The apparent discrepancy can be accounted for by the persistence of store loading, which can last for several minutes (Pozzo-Miller et al 2000), much longer than the Ca^{2+}-dependent IP_3R inactivation that recovers with a time constant of seconds.

4.3 Burst- and mGluR-induced Ca^{2+} signals and synaptic plasticity

It has been shown recently that LTP and LTD of AMPA-mediated transmission can be induced in a manner dependent on postsynaptic bursts of APs and mGluR activation, respectively, in DA neurons (Bellone & Luscher 2005; Liu et al 2005), although other forms of plasticity have been reported using different induction protocols (Gutlerner et al 2002; Ungless et al 2001). It is interesting to note that a cooperative Ca^{2+} signal, produced by Ca^{2+} spikes paired with weak, sustained activation of mGluRs, mediates the synaptic plasticity in Purkinje neurons responsible for cerebellar motor learning (Doi et al 2005; Wang et al 2000). Our preliminary data show that a similar induction protocol, in which weak, sustained mGluR activation is followed by a burst of APs to generate a supralinear Ca^{2+} transient (see Fig. 3.7), induces LTP of NMDA-mediated transmission onto DA neurons (unpublished observation). Thus, an increase in IP_3 tone may shift the balance of DA neuron plasticity toward LTP by selectively amplifying burst-induced Ca^{2+} signals.

DA neurons also receive other neurotransmitter inputs activating PI-coupled metabotropic receptors. These include noradrenergic, cholinergic, and peptidergic (corticotropin-releasing factor [CRF] and orexin) inputs, which can all act through volume transmission, i.e., through a rise in extracellular tone, and are involved in behavioral arousals (Aston-Jones & Cohen 2005; Austin et al 1997; Harris & Aston-Jones 2006; Kobayashi et al 2004; Zoli et al 1998). An elevated

arousal state, such as that resulted from being exposed to stress or a novel environment, facilitates the reinstatement of drug-seeking behaviors and psychostimulant-induced behavioral sensitization (Badiani et al 1995; Lu et al 2003). Interruption of these neurotransmitter systems, such as blockade of mAChRs, CRF receptors and orexin receptors, inhibits reward-related learning and suppresses reinstatement of drug seeking behaviors (Boutrel et al 2005; Shaham et al 1998; Sharf & Ranaldi 2006). Furthermore, a recent study showed that orexin facilitated cocaine-induced LTP in DA neurons (Borgland et al 2006). Therefore, by promoting the potentiation of glutamatergic transmission onto DA neurons, the differential regulation of Ca^{2+} signals described in this study may contribute to enhanced reinforcement learning when animals are placed in behaviorally arousing environments.

4.4 Amphetamine regulation of Ca^{2+} signaling in DA neurons: a potential mechanism for psychostimulant addiction

Dopaminergic nuclei are believed to be the site that mediates the induction of enduring behavioral adaptations in psychostimulant addiction (Jones & Bonci 2005; Kauer 2004; Vanderschuren & Kalivas 2000). Psychostimulant-induced inhibition of LTD and enhancement of LTP in DA neurons have been proposed to be candidate cellular mechanisms underlying these adaptations. Since mGluR activation and post-synaptic APs have been implicated in certain forms of LTD (Bellone & Luscher 2005) and LTP (Liu et al 2005) in DA neurons, respectively, it is plausible that PI-coupled receptor-induced differential regulation of these two types of Ca^{2+} signals serves as a unique mechanism that mediates the effect of psychostimulants on strengthening glutamatergic synapses in DA neurons. Indeed, this hypothesis is supported by evidences showing that activation of mGluRs (Dunn et al 2005; Kim & Vezina 1998), α1Rs (Auclair et al 2004; Drouin et al 2002; Mohammed et al 1986), and mAChRs (Basile et al 2002; Fink-Jensen et al 2003) are all implicated in psychostimulant-induced locomotor sensitizing or reinforcing effects.

Psychostimulant amphetamine has been shown to increase extracellular glutamate (Xue et al 1996) and acetylcholine (Rada et al 2007) in the ventral midbrain. In addition to stimulating multiple neurotransmitter systems through

long loops, amphetamine also directly regulates DA neuron activity by releasing catecholamines within the somatodendritic areas (Seiden et al 1993). A long-held view about local pharmacological action of amphetamine is that it exerts primarily inhibitory effect on DA neuron firing via D2 autoreceptors (Aghajanian & Bunney 1977). In addition to D2R-mediated inhibition, recent findings suggested that amphetamine-induced NE (Shi et al 2000) and DA (Paladini et al 2001) release could also excite DA neurons by activating α1ARs, which suppresses phasic mGluR-induced Ca^{2+} release and subsequent SK channel activation (Paladini et al 2001). In the present study, we found that amphetamine-induced D2R and α1AR activation both contributed to the enhancement of burst-induced Ca^{2+} signals. We showed that amphetamine preferentially suppressed tonic firing with only a small inhibitory effect on iGluR-induced bursts, probably because the iGluR-mediated excitatory drive can largely overcome the D2R-mediated inhibition. A recent report showed that cocaine has similar differential effects on basal firing and evoked bursts *in vivo* (Almodovar-Fabregas et al 2002). This D2R-mediated selective inhibition of basal firing removes Ca^{2+}-dependent inactivation of IP_3Rs, thereby augmenting CICR. Our results suggest that amphetamine-induced activation of D2Rs and α1ARs played concerted roles in boosting burst-induced Ca^{2+} signals. This is consistent with behavioral studies showing that blocking either D2Rs (Hamamura et al 1991; Karler et al 1990; Kuribara 1996; Meng et al 1998; Ujike et al 1989) or α1ARs (Darracq et al 1998; Dickinson et al 1988)inhibits amphetamine-induced locomotor sensitization

CHAPTER 5: CONCLUSION

Previous studies suggest that potentiated synaptic transmission between glutamatergic input and midbrain DA neurons constitutes one of the cellular mechanisms mediating reward-related reinforcement learning and drug addiction (Jones & Bonci 2005; Kauer 2004). It has been shown that Ca^{2+} signals associated with post-synaptic APs and mGluR activation produce LTP (Liu et al 2005; Pu et al 2006) and LTD (Bellone & Luscher 2005), respectively in DA neurons. In this dissertation, we found that the 'LTD signal', mGluR-mediated Ca^{2+} responses, and the 'LTP signal', AP-induced Ca^{2+} transients, are differentially regulated by sustained activation of PI-coupled receptors in DA neurons.

Furthermore, we found that repetitive APs that mimic physiological basal firing of DA neurons inhibit AP-induced Ca^{2+} transients by causing inactivation of IP_3Rs. In line with this observation, we showed that amphetamine, which selectively suppresses basal firing in DA neurons via activating D2 autoreceptors, may augment burst-induced Ca^{2+} signals by both activation of D2Rs and production of IP_3.

Our findings led to a novel hypothesis of how addictive drugs, especially psychostimulant amphetamine, modulate plasticity at DA neuron synapses (Fig. 5.1). In this scenario, amphetamine elevates intracellular IP_3 concentration via

activating mGluRs, α1ARs, and mAChRs through long loop and/or by stimulating α1ARs through locally inducing NE and DA release. The increased intracellular IP_3 tone then suppresses LTD-associated mGluR-induced Ca^{2+} signals while enhancing LTP-associated AP-induced Ca^{2+} transients. In addition, amphetamine selectively inhibits basal firing of DA neurons by activation of D2 autoreceptors, thus further augments burst of AP-induced Ca^{2+} signals. This differential regulation of mGluR- and AP-induced Ca^{2+} signals may shift the balance of synaptic plasticity toward potentiation, leading to a drug addictive state at behavioral level.

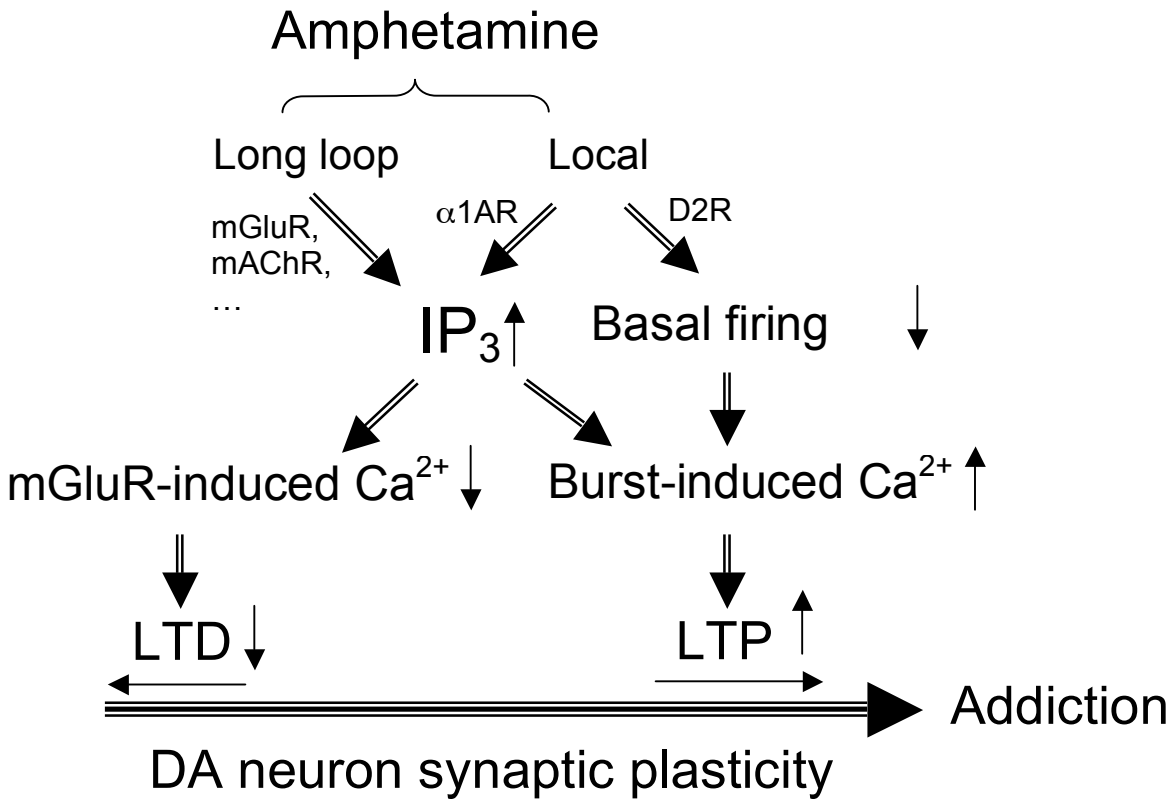

Figure 5.1 Proposed cellular mechanisms mediating amphetamine addiction.

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Vita

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